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TITLE: A Targeted Mulifunctional Platform for Imaging and Treatment of Breast Cancer and Its Metastases Based on Adenoviral Vectors and Magnetic Nanoparticles

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14. ABSTRACT
Nanotechnology holds many promises for the imaging and treatment of breast cancer. In particular, magnetic nanoparticles can be utilized for tumor imaging via magnetic resonance imaging (MRI) techniques and tumor treatment by heating after exposure to an alternating magnetic field. However, selective targeting of the nanoparticles to the tumor cells needs to be accomplished before the imaging or therapy can be successful. In this respect, adenoviral (Ad) vectors for gene therapy have made great progress in achieving successful tumor targeting after their intravenous administration. We therefore hypothesized that coupling magnetic nanoparticles to targeted Ad vectors would allow the selective localization of these particles to the tumor, thereby making imaging and therapy possible. We herein identified that coupling gold nanoparticles to the Ad capsid protein hexon does not perturb vector infectivity or retargeting efficiency in vitro. Furthermore, we identified that available magnetic nanoparticles did not provide enough contrast to be suitable for Ad-mediated delivery and subsequent MRI. We therefore pursued the coupling of quantum dots (QDs) as an alternative imaging-method, and demonstrated that Ad vectors can deliver these intracellularly in breast cancer cells. However, QDs abrogated transgene expression of the Ad vectors by reasons not related to size or coupling chemistry. Future research will aim at identifying the reasons for transgene abrogation.

15. SUBJECT TERMS
Nanotechnology; gene therapy; targeting; breast cancer

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INTRODUCTION

Nanotechnology holds great promise for the imaging and treatment of breast cancer. In this regard, magnetic nanoparticles have utilies in cancer imaging via Magnetic Resonance Imaging (MRI) technologies, and utilities in cancer treatment via hyperthermia induction upon exposure to an alternating magnetic field. However, success of both utitilities will greatly depend on the ability to target these magnetic nanoparticles selectively to tumors. In this regard, adenoviral gene therapy vectors have made great progress in selectively targeting tumors, both *in vitro* and *in vivo*. Of note, we have previously linked metal (gold) nanoparticles to adenoviral (Ad) vectors, and have demonstrated that gene transfer and vector tumor targeting are not negatively affected by this process. This feasibilizes future combination of nanotechnology-mediated imaging and treatment of cancer with gene therapy. We therefore herein proposed to explore the paradigm of coupling magnetic nanoparticles to targeted Ad vectors, thereby creating novel multifunctional particles that can simultaneously target, image and treat breast cancer. During the first period of this project it was determined that commercially available magnetic nanoparticles with the appropriate surface chemistry needed for coupling to Ad vectors did not display sufficient MRI-contrast, as analyzed in our small animal 9.4 T MRI scanner. We therefore explored in the remaining period whether magnetic nanoparticles produced by a collaborator, Dr. David E. Nikles, University of Alabama, Tuscaloosa, would provide better contrast abilities. In addition, we pursued the coupling of Quantum Dots (QDs) as an alternative to magnetic nanoparticles, to facilitate light-based imaging for tumor detection. In combination with gene therapy applications, this will provide a multi-faceted approach for the imaging and treatment of breast cancer as an alternative to the initally proposed MRI-based system.

BODY

The objectives of this project were as follows:

#1: Couple magnetic nanoparticles to adenoviral vectors using strategies retaining virus infectivity and targeting ability;

#2: Analyze targeting and imaging capabilities of developed multifunctional platforms in a murine model of breast cancer.

We will herein summarize our efforts to reach the objectives as stated above.

#1: <u>Couple magnetic nanoparticles to adenoviral vectors using strategies retaining virus infectivity and targeting ability</u>

A. Hexon is the best adenovirus capsid location for coupling of nanoparticles

As described in our annual report, we started this project by developing a system that could be used to couple nanoparticles (NPs) to the viral backbone without interfering with the virus infection and retargeting process. To achieve this specific coupling, we genetically manipulated Ad capsid proteins to introduce NP binding sites at locales not involved in the vector infection and targeting pathway. In particular, we employed a high-affinity interaction between a sequence of six-histidine amino acid residues genetically incorporated into various Ad capsid proteins (fiber fibritin, pIX and hexon) and Nickel (II) Nitrilotriacetic acid on the surface of NPs. Our results demonstrate the selective self-assembly of NPs and Ad vectors into the envisioned multifunctional platforms. Of the various capsid locations, the hexon location proved most efficient in NP binding, resulting in 56 bound NPs per virion. Importantly, compared to previously employed coupling strategies, this selective assembly did not negatively affect targeting of Ad to specific cells. At the time of our annual report, these data had been submitted for publication to the journal 'Small', which now has been accepted and published (see reportable outcome 1). Of note, 'Small' has the second highest impact factor of nanotechnology journals (6.024), illustrating the relevance of our coupling method to the scientific community. The data have also been presented in 1 article and 6 abstracts for scientific meetings (see reportable outcomes 3, 4-9) and have served as preliminary data in several grant proposals (see reportable outcomes 10-12). Finally, we have presented this data in invited lectures, both within UAB and outside of the institution (see reportable outcomes 13-17).

B. Employed magnetic nanoparticles lack sufficient MRI contrast to serve as imaging moieties for breast cancer

As mentioned in the annual report, we discovered that commercially available magnetic nanoparticles from Miltenyi were not suitable for the envisioned application that entails coupling to Ad vectors and targeting to breast cancer cells, due to insufficient MRI contrast. We utilized our connections with Dr. David E. Nikles at the University of Alabama Center for Materials for Information Technology (MINT Center) and obtained samples of alternative magnetic nanoparticles:

1. $Fe_1Ni_4Pt_5$
2. $Fe_4Ni_1Pt_3$
3. $Fe_4Ni_1Pt_5$
4. $Fe_1Ni_1Pt_2$
5. FePtAg

We tested these nanoparticles for MRI-contrast properties in our 9.4 T small animal MRI scanner, in different dilutions. Results demonstrated that only in the highest concentrations of 1 mg/mL sufficient contrast could be obtained. When calculating the number of nanoparticles that can be coupled to Ad vectors and how many vectors that can bind to breast cancer cells, it was concluded that not enough nanoparticles could be delivered to reach the 1 mg/mL concentration. We therefore decided to not pursue the coupling of currently available magnetic nanoparticles to Ad vectors anymore, but instead focus on alternative imaging agents to be coupled to Ad vectors.

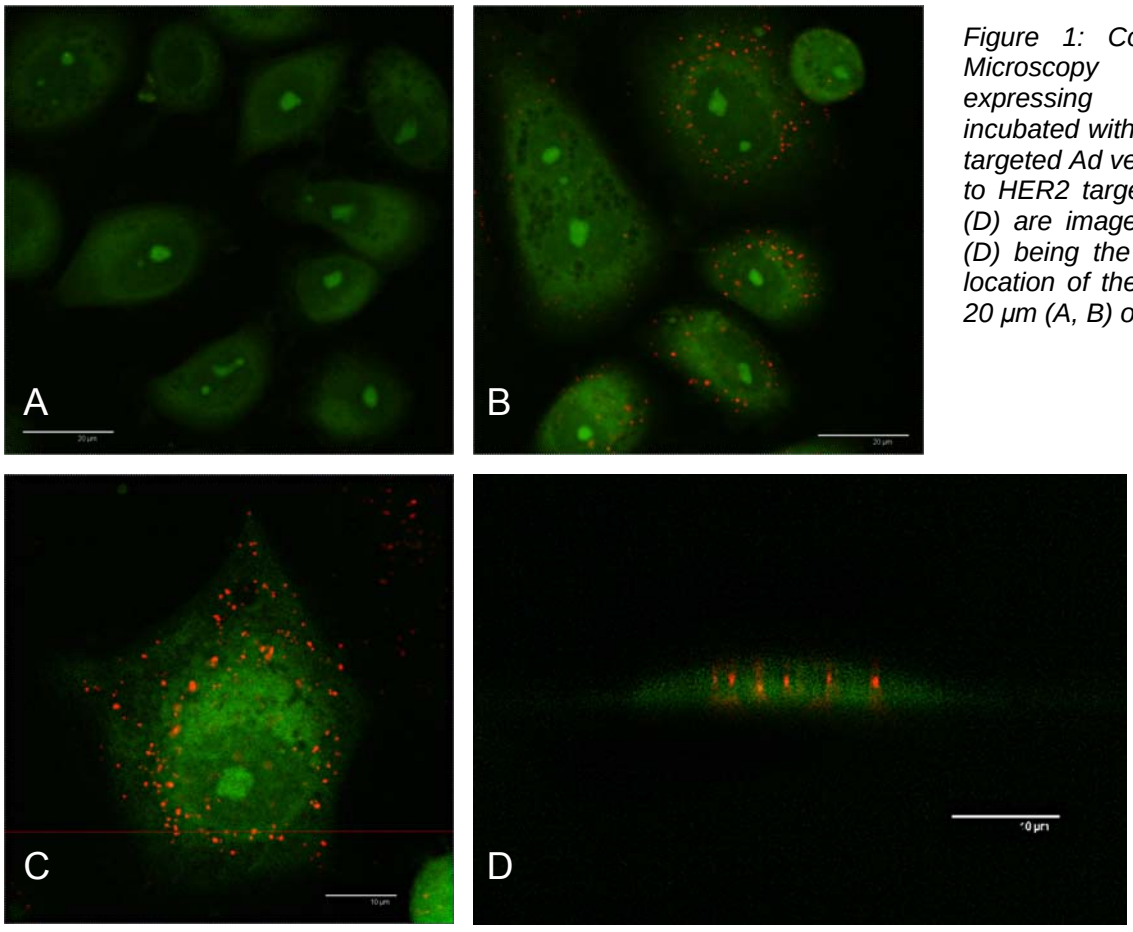

Figure 1: Confocal Laser Scanning Microscopy of AU-565 HER2-expressing breast cancer cells, incubated with (A) QDs coupled to non-targeted Ad vectors, (B-D) QDs coupled to HER2 targeted Ad vectors. (C) and (D) are images of the same cell, with (D) being the XZ image of (C) at the location of the line (arrow). Scale bars 20 µm (A, B) or 10 µm (C, D).

C. Quantum Dots are alternative imaging agents to be coupled to targeted Ad vectors

As an alternative to magnetic nanoparticles that can be utilized for MRI-based imaging, we decided to pursue the use of Quantum Dots (QDs) for light-based imaging of breast cancer. The coupling method we employed to link the two components together was based on Ad vectors expressing biotin in the hexon protein, and QDs labeled with streptavidin. In this regard, we have shown in our annual report that we could indeed couple QDs to targeted Ad vectors using this strategy, resulting in selective uptake in c-erbB2 expressing MDA-MB-361 and AU-565 breast cancer cells (Figure 1 on page 5). This indicated the feasiblity of our chosen approach to construct a targeted multifunctional system for the imaging and treatment of breast cancer.

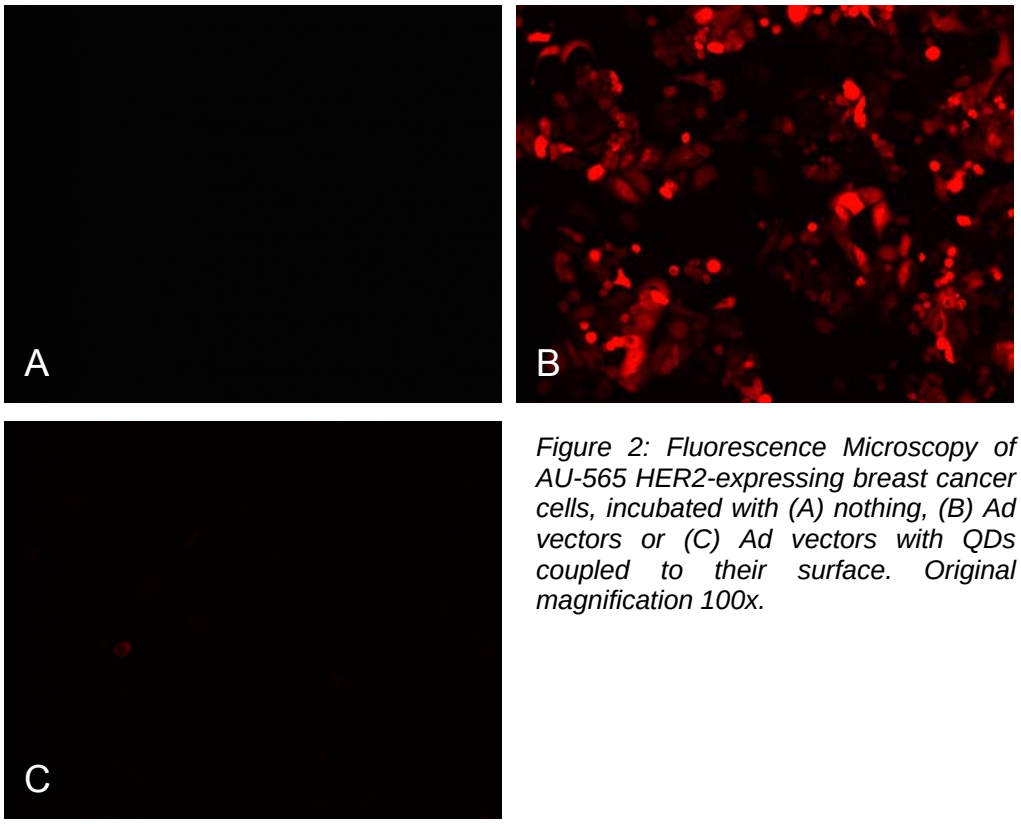

Figure 2: Fluorescence Microscopy of AU-565 HER2-expressing breast cancer cells, incubated with (A) nothing, (B) Ad vectors or (C) Ad vectors with QDs coupled to their surface. Original magnification 100x.

D. Quantum Dots abolish transgene expression when coupled to Ad vectors

Since the annual report we have continued our investigation of targeted QD-Ad complexes, and our first task was to confirm that coupling of QDs to Ad vectors did not interfere with gene expression. For this, AU-565 cells were non-infected, infected with targeted Ad by itself, or infected with targeted Ad labeled with QDs. The utilized Ad vectors that have a biotin acceptor peptide in the hexon protein encode red fluorescence protein (RFP) as a transgene, so after 24 h RFP expression was examined using standard fluorescence microscopy. As expected, uninfected cells did not express any RFP and cells infected with targeted Ad expressed high levels of RFP (Figure 2A,B). However, unexpectedly, when QDs were coupled to the Ad vectors, transgene expression was completely abolished (Figure 2C).

One potential explanation of the lack of observed RFP expression was that the QDs would quench the emitted photons and emit at 655 nm (their emission peak), which would not be observed using our filters. We therefore harvested the cells by lysing them in Laemmli sample buffer supplied with protease inhibitors, and analyzed RFP expression using Western Blot. This analysis confirmed the absence of RFP expression in uninfected cells (Figure 3, lane 1), the presence of RFP expression in ells infected with Ad vectors (Figure 3, lane 2), and the absence of RFP expression in cells infected with QD-labeled Ad vectors (Fig 3, lane 3).

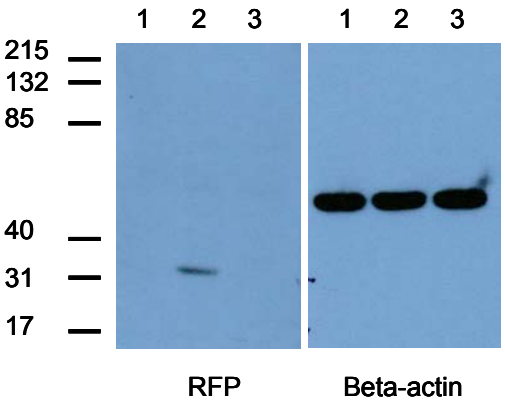

Figure 3: Western Blot of cell lysates of AU-565 HER2-expressing breast cancer cells, incubated with (1) nothing, (2) Ad vectors or (3) Ad vectors with QDs coupled to their surface. Blots were stained with an antibody recognizing red fluorescence protein (RFP) or beta-actin as a loading control.

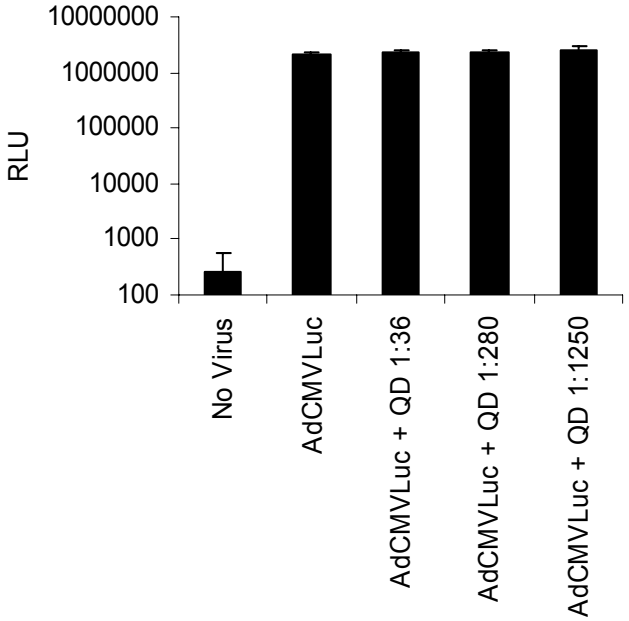

Figure 4: Non-binding Quantum Dots (QDs) do not have a negative effect on transgene expression mediated by Ad infection. Cells were incubated with an Ad vector expressing luciferase under transcriptional control of the CMV promoter. Vectors were incubated with different amounts of QDs: 36, 280 or 1250 QDs per virion.

E. Lack of transgene expression not due to toxicity of Quantum Dots

Our next hypothesis was that the QDs would have a negative effect on cell viability, thereby inhibiting transgene expression in infected cells. To test this hypothesis, we utilized an adenoviral vector expressing luciferase that did not have a biotin tag in the hexon protein, nor anywhere else in the capsid; incubated the vector with or without QDs, after which AU-565 cells were transduced for 3 hours. Transgene expression was measured after 24 hours. We did not observe any differences in transgene expression in the different samples, irrespective of how many QDs we added to the vectors (36, 280 or 1250 QDs per virus; Figure 4). This indicated that QDs did not have an aspecific toxic effect on transduced cancer cells and disproved this particular hypothesis.

F. Lack of transgene expression not due to coupling method or size of Quantum Dots

Our previous results, as published in *Small,* demonstrated that when we coupled 1.8 nm-sized gold nanoparticles to Ad vectors, transgene expression of the vector was retained. In the data presented above, we demonstrate that 35 nm-sized QDs, when coupled to Ad veoctors, abolish transgene expression. Thus, there is a considerable size difference between the two types of nanoparticles used, in addition to the difference in metal composition (gold vs cadmium-selenium). Furthermore, we used a different coupling method to attach the nanoparticles to the vector: in our published study we utilized gold nanoparticles labeled with a Ni-NTA group to couple to a six-histidine tag in hexon, whereas we herein utilized streptavidin-labeled QDs to couple to a biotin tag in hexon. To determine whether size or coupling method would have an effect on the level of transgene expression, we obtained gold nanoparticles of different sizes (5, 20 and 40 nm in diameter) with a streptavidin molecule on their surface (Nanocs Inc, New York, NY) and coupled them to Ad vectors with a biotin-tag in hexon, like we have coupled QDs to Ad vectors. We incubated the complexes, as before, with c-erbB2 expressing AU-565 breast cancer cells, and examined RFP expression after 24 hours. Like in our previous experiments, QDs abolished transgene expression in infected cells (Figure 5, second column). However, coupled gold nanoparticles did not have an effect on transgene expression (Figure 5, third, fourth and fifth column), indicating that neither the size of the QDs nor the utilized coupling method was responsible for the inhibition of transgene expression.

G. Endosome disruption to aid in endosomal release of Ad vectors does not restore transgene expression

It has been described in literature that QDs tend to aggregate inside living cells, and are trapped into organelles such as endo- and lysosomes.[1] We therefore hypothesized that QDs coupled to Ad vectors would also aggregate after intracellular uptake, trapping the vectors in the endosomes and preventing endosomal escape with subsequent translocation to the nucleus. This would prevent the genetic material to be taken up and subjected to the cellular transcription and translation machinery. We therefore decided to test the effects of the presence of the lysosomotropic agent chloroquine, which has endosome disrupting properties,[2] thereby hypothetically aiding in endosomal escape of trapped virions. We analyzed RFP expression of infected cells using fluorescence microscopy at days 1, 2, 3, 6 and 7 days after infection, but found no significant effect on transgene expression in any of the samples (day 2 data shown in Figure 6, other days not shown because of similar results).

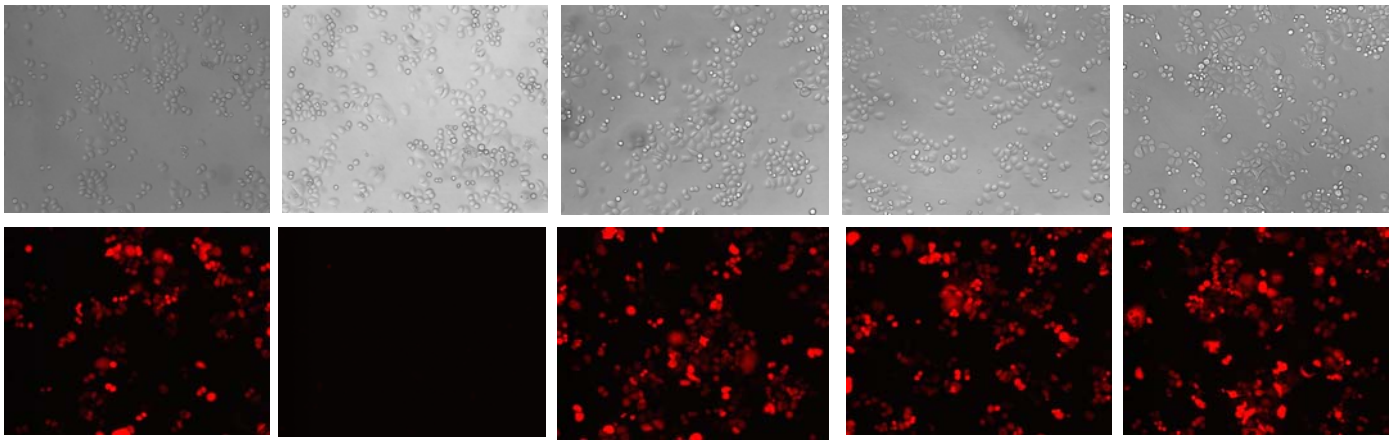

Figure 5: Gold nanoparticles (AuNPs) of either 5, 20 or 40 nm in diameter, when coupled to Ad vectors via a biotin-streptavidin bridge, do not interfere with RFP expression in infected cells, unlike Quantum Dots (QDs). Top panels indicate the presence of cells in all samples, as observed with light microscopy, whereas bottom panels indicate the level of fluorescence observed in the rhodamine channel. Original magnification 100x.

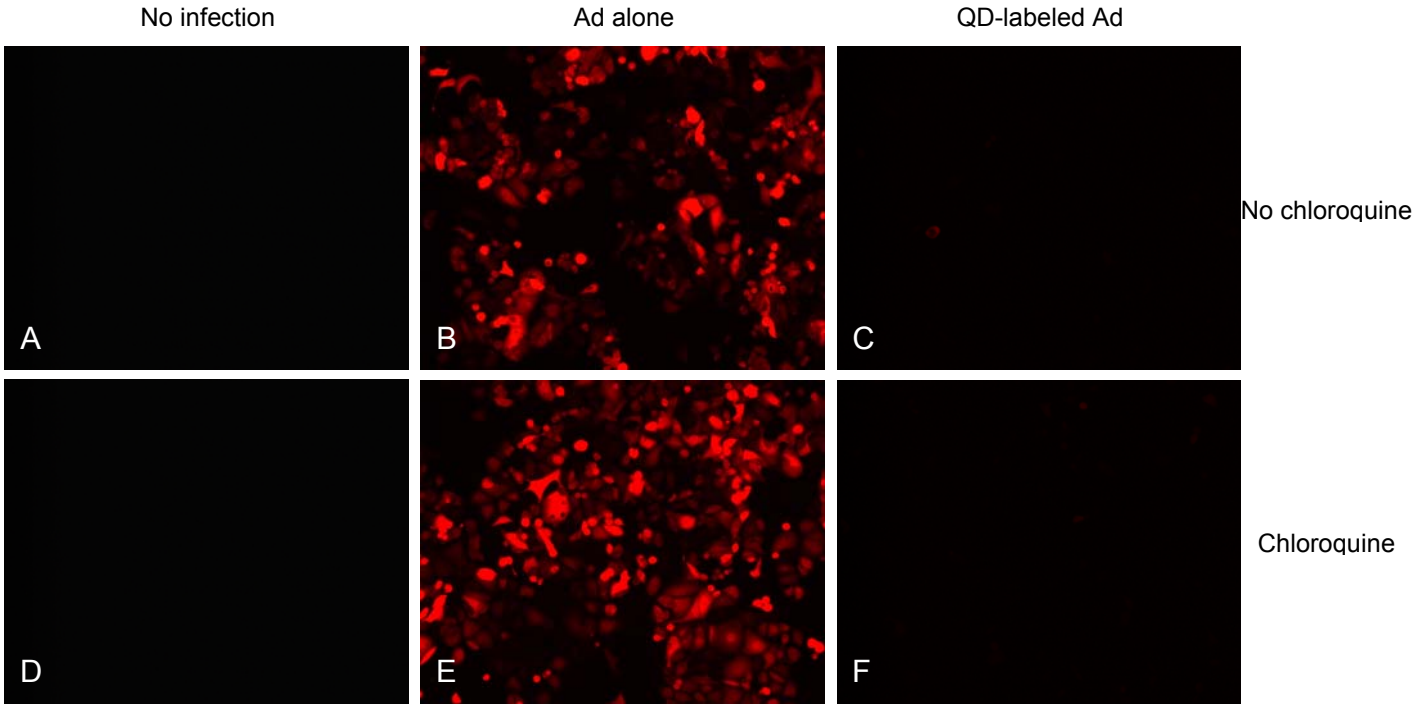

Figure 6: Presence of 100 uM chloroquine does not reinstate transgene expression in cells infected with QD-labeled Ad vectors. Original magnification 100x.

At this point it is unclear whether chloroquine can aid in endosomal release of QDs and thus Ad vectors – no literature could be found on this topic. However, Duan and Nie described the endosomal release of QDs by coating the nanoparticle with an endosome-disrupting polymer consisting of polyethylene glycol (PEG) grafted polyethylenimine (PEI-*g*-PEG). Future plans therefore include the coupling of QDs coated with this polymer to Ad vectors, and analyzing transgene expression after cellular infection with these complexes.

#2: Analyze targeting and imaging capabilities of developed multifunctional platforms in a murine model of breast cancer

Objective #2 was not pursued due to the obstacles encountered under objective #1.

KEY RESEARCH ACCOMPLISHMENTS

- We analyzed the T1 and T2 values of our commercially obtained magnetic nanoparticles at 9.4 T, establishing that these particles are a T2 contrast agent.
- We successfully identified a capsid location (hexon) for coupling metal (gold) nanoparticles to adenoviral vectors that does not interfere with targeted gene delivery.
- We ascertained that the commercially available magnetic nanoparticles do not provide enough T2 magnetic contrast when targeted to cells *in vitro* by adenoviral vectors.
- We discovered that nanoparticles derived from magnetic materials, as developed by Dr. Nikles et al, also lack sufficient MRI contrast for adenoviral vector based targeting.
- We demonstrated successful targeting and fluorescence-based imaging of metal (quantum dot) nanoparticles on adenoviral vectors to breast cancer cells *in vitro*, using the sCAR-C6.5 adapter molecule.
- We discovered that quantum dot-labeled adenoviral vectors are unable to express their transgene in transduced breast cancer cells.
- We identified that the lack of transgene expression is not due to the size or coupling method of quantum dots to adenoviral vectors, but rather due to other intrinsic properties such as surface chemistry.

REPORTABLE OUTCOMES

Manuscripts:

1. An Adenoviral Platform for Selective Self-Assembly and Targeted Delivery of Nanoparticles. V. Saini, D.V. Martyshkin, S.B. Mirov, A. Perez, G. Perkins, M.H. Ellisman, H. Wu, L. Pereboeva, A. Borovjagin, D.T. Curiel, **M. Everts**. *Small* 4(2):262-269, 2008
2. Importance of Viruses and Cells in Cancer Gene Therapy. V. Saini, J.C. Roth, L. Pereboeva, **M. Everts**. *Advances in Gene, Molecular and Cell Therapy* 1(1): 30-43, 2007
3. Targeting Nanoparticles to Tumors using Adenoviral Vectors. V. Saini, M.R. Enervold, A. Perez, A. Koploy, G. Perkins, M.H. Ellisman, H.N. Green, S.B. Mirov, V.P. Zharov, **M. Everts**. *NSTI-Nanotech 2007*, Vol. 2: 321-324, 2007

Abstracts (poster presentations):

4. Adenoviral Platform for Selective Assembly and Targeted Delivery of Gold Nanoparticles to Tumor Cells; V. Saini, A. Perez, A. Koploy, G. Perkins, M.H. Ellisman, D.E. Nikles, D.T. Johnson, D.T. Curiel, **M. Everts**. Presented as poster at the Keystone Meeting "Nanotechnology in Biomedicine", February 11- 26, 2007. *Note: Vaibhav Saini was the sole recipient of a Keystone Travel Award to attend this meeting.*
5. Targeting Nanoparticles to Tumors using Adenoviral Vectors. V. Saini, M.R. Enervold, A. Perez, A. Koploy, G. Perkins, M.H. Ellisman, H.N. Green, S.B. Mirov, V.P. Zharov, **M. Everts**. Presented as poster at the NSTI-Nanotech 2007 meeting, May 20-24, 2007
6. Determining Parameters for Using Gold Nanoparticles for Hyperthermia Treatment in Tumor Cells. V.D. Towner, V. Saini, D.V. Martyshkin, S.B. Mirov, **M. Everts**. Presented as poster in context of the UAB McNair Summer Research Program, July 24, 2007
7. J.M. Warren, R.G. Beam, V. Saini, M.R. Enervold, A. Perez, G. Perkins, M.H. Ellisman, **M. Everts**. Targeting Nanoparticles to Tumors using Adenoviral Vectors. Presented as poster on the UAB Comprehensive Cancer Center, October 29, Birmingham, AL. *Note: Maaike Everts won 2nd place for the John R. Durant Award for Excellence in Cancer Research, Junior Faculty category.*
8. V. Saini, D.V. Martyshkin, S.B. Mirov, V.D. Towner, H. Wu, L. Pereboeva, A. Borovjagin, G. Perkins, M.H. Ellisman, D.T. Curiel, **M. Everts**. Adenovirus as a Platform for Selective Assembly and Targeted

Delivery of Gold Nanoparticles to Tumor Cells. American Association for Cancer Research (AACR) Annual Meeting, San Diego, CA, April 12-16, 2008

9. J.M. Warren, R.G. Beam, V. Saini, **M. Everts.** Targeting Quantum Dots to Tumors using Adenoviral Vectors. American Association for Cancer Research (AACR) Annual Meeting, San Diego, CA, April 12-16, 2008

Grant submissions in which obtained data was used as preliminary data:

10. 1R01 CA125357-01 (**Everts**/Curiel); 4/01/2008 – 3/31/2013; NIH/NCI; annual $499,063/total $2,248,588. Magnetic Nanoparticles on Targeted Adenovirus for Imaging and Therapy of Cancer. *Result: 39.9 percentile*
11. UAB Breast SPORE pilot grant (**Everts**); 3/01/2008 – 2/25/2009; annual $50,000. Radioactive Nanoparticles on Adenoviral Vectors for Breast Cancer Imaging and Therapy. *Pending*
12. Komen for the Cure, Career Catalyst Award (**Everts**); 4/01/2008 – 3/31/2009; annual $145.491. Radioactive nanoparticles on adenoviral vectors for multipronged breast cancer therapy. *Pending*

Invited lectures in which obtained data was presented:

13. Multifunctional Potential of Nanotechnology and Gene Therapy for Cancer. M. Everts. Invited lecture at the University of Alabama at Birmingham Gene Therapy Center Research Symposium, Birmingham, AL, October 12th, 2007
14. Multifunctional Potential of Nanotechnology and Gene Therapy for Cancer. M. Everts. Invited lecture at the University of Alabama, Tuscaloosa, AL, September 13th, 2007
15. Multifunctional Potential of Nanotechnology and Gene Therapy for Cancer. M. Everts. Invited lecture at Emory-Georgia Tech Nanotechnology Center for Personalized and Predictive Oncology, April 27th, 2007
16. Adenoviral Vectors: Golden Opportunities for Multifunctional Nanoparticles. M. Everts. Invited lecture for the Heamatology/Oncology Seminar Series, UAB, January 22nd, 2007
17. Adenoviral Vectors: Golden Opportunities for Multifunctional Nanoparticles. M. Everts. Invited lecture at the University of the Pacific, Stockton, CA, November 7th, 2006

CONCLUSION

We have demonstrated that we can specifically couple gold NPs to Ad vectors without compromising their infectivity or retargeting efficacy – a major improvement over previous unspecific coupling strategies. This finding was published in the prestigious nanotechnology journal 'Small'. Our aim was to couple magnetic nanoparticles to Ad vectors, but due to lack of sufficient contrast of both commercially available magnetic nanoparticles as well as newly developed nanoparticles by a collaborator, we focused our efforts on coupling quantum dots to adenoviral vectors. We have demonstrated successful targeting of quantum dot-labeled Ad vectors to breast cancer cells *in vitro*, resulting in their intracellular accumulation. However, by coupling quantum dots to the vectors their transgene expression was abolished. We have determined that this abolishment is not due to size of the nanoparticles, the coupling chemistry, nor toxicity. Potentially the lack of transgene expression is a consequence of surface chemistry of the quantum dots and their subsequent entrapment in endosomes – a hypothesis that we will pursue in future research plans.

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1 Duan H, Nie S. Cell-penetrating quantum dots based on multivalent and endosome-disrupting surface coatings. *J Am Chem Soc* 2007; 129: 3333-3338.

2 Shiraishi T, Nielsen PE. Enhanced delivery of cell-penetrating peptide-peptide nucleic acid conjugates by endosomal disruption. *Nature Protocols* 2006; 1: 1-4.

APPENDICES

Manuscripts:

1. V. Saini, D.V. Martyshkin, S.B. Mirov, A. Perez, G. Perkins, M.H. Ellisman, H. Wu, L. Pereboeva, A. Borovjagin, D.T. Curiel, **M. Everts**. An Adenoviral Platform for Selective Self-Assembly and Targeted Delivery of Nanoparticles. *Small*, 2008; 4: 262-269

2. Saini, V., J.C. Roth, L. Pereboeva, **M. Everts**. Importance of viruses and cells in cancer gene therapy. *Advances in Gene, Molecular and Cell Therapy*, 2007, 1(1):30-43

3. Targeting Nanoparticles to Tumors using Adenoviral Vectors; V. Saini, M.R. Enervold, A. Perez, A. Koploy, G. Perkins, M.H. Ellisman, H.N. Green, S.B. Mirov, V.P. Zharov, **M. Everts**. *NSTI-Nanotech, 2007,* 2:321-324

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5. V. Saini, D.V. Martyshkin, S.B. Mirov, V.D. Towner, H. Wu, L. Pereboeva, A. Borovjagin, G. Perkins, M.H. Ellisman, D.T. Curiel, **M. Everts**. Adenovirus as a Platform for Selective Assembly and Targeted Delivery of Gold Nanoparticles to Tumor Cells. American Association for Cancer Research (AACR) Annual Meeting, San Diego, CA, April 12-16, 2008 – accepted for poster presentation.

6. J.M. Warren, R.G. Beam, V. Saini, **M. Everts.** Targeting Quantum Dots to Tumors using Adenoviral Vectors. American Association for Cancer Research (AACR) Annual Meeting, San Diego, CA, April 12-16, 2008 – accepted for poster presentation.

Viral vectors

DOI: 10.1002/smll.200700403

An Adenoviral Platform for Selective Self-Assembly and Targeted Delivery of Nanoparticles

*Vaibhav Saini, Dmitri V. Martyshkin, Sergei B. Mirov, Alex Perez, Guy Perkins, Mark H. Ellisman, Victoria D. Towner, Hongju Wu, Larisa Pereboeva, Anton Borovjagin, David T. Curiel, and Maaike Everts**

***M**etallic nanoparticles (NPs) can be used for the diagnosis, imaging, and therapy of tumors and cardiovascular disease. However, targeted delivery of NPs to specific cells remains a major limitation for clinical realization of these potential treatment options. Herein, a novel strategy for the specific coupling of NPs to a targeted adenoviral (Ad) platform to deliver NPs to specific cells is defined. Genetic manipulation of the gene therapy vector is combined with a specific chemical coupling strategy. In particular, a high-affinity interaction between a sequence of six-histidine amino acid residues genetically incorporated into Ad capsid proteins and nickel(II) nitrilotriacetic acid on the surface of gold NPs is employed. The selective self-assembly of gold NPs and Ad vectors into multifunctional platforms does not negatively affect the targeting of Ad to specific cells. This opens the possibility of using Ad vectors for targeted NP delivery, thereby providing a new type of combinatorial approach for the treatment of diseases that involves both nanotechnology and gene therapy. ■Please check shortened abstract■*

Keywords:
- cell recognition
- gene expression
- nanoparticles
- self-assembly
- viruses

1. Introduction

Nanotechnology is revolutionizing the field of biomedicine. In this regard, metallic nanoparticles (NPs), such as quantum dots (QDs), magnetic NPs, and gold NPs (AuNPs), can be used for tissue welding, gene regulation, intracellular environment studies, diagnosis, imaging, and hyperthermic tumor-cell killing.[1–6] However, targeted delivery of NPs to specific cells is a major impediment for the successful clinical utilization of the multiple treatment opportunities provided by nanotechnology. We previously hypothesized that adenoviral (Ad) vectors, which are used as targeted vectors for gene therapy,[7] might provide a suitable platform for target-specific delivery of NPs. This would allow a combination of the proven gene-delivery capacity of Ad vectors with the imaging and therapeutic potential of NPs for the treatment of disease. To this end, we have demonstrated that NPs can be coupled to Ad vectors using a nonspecific coupling method. However, nonspecific NP cou-

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pling to Ad vectors in high NP:Ad ratios resulted in abrogation of Ad vector infectivity in target cells.[8]

To circumvent this problem and thereby extend the paradigm of NP targeting via an Ad vector platform, we herein hypothesize that *specific* AuNP coupling to Ad vectors prevents the detrimental effects on Ad vector infectivity and targeting observed with nonspecific NP coupling. To achieve specific coupling, we genetically manipulated Ad capsid proteins to introduce NP binding sites at locales not involved in the vector infection and targeting pathway. Validation of this hypothesis paves the way towards realization of a multifunctional nanoscale system that combines gene therapy and nanotechnology approaches for the targeting and treatment of disease. In addition, it serves as a proof of principle that Ad vectors can serve as a platform for specific self-assembly of multiple components.

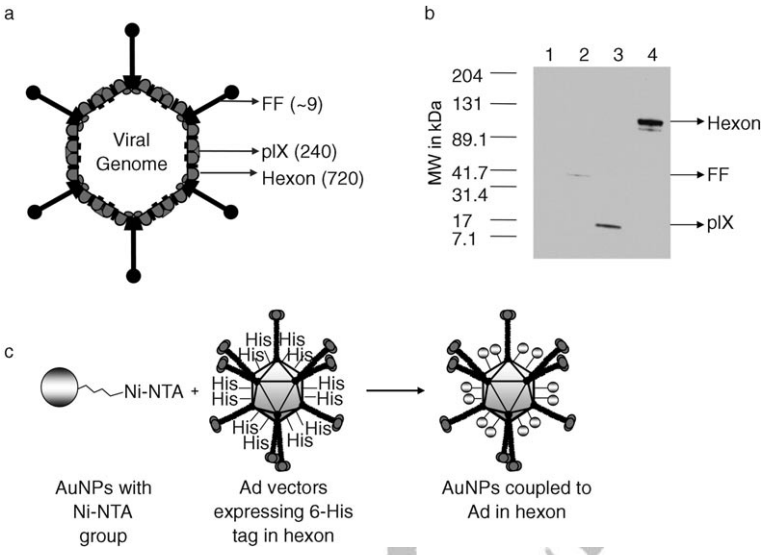

Figure 1. NPs can be specifically coupled to distinct Ad capsid locations. a) Schematic representation of the location of various structural proteins in the Ad capsid. The Ad vectors used in this study contain a 6-His tag genetically incorporated in either modified fiber (FF mosaic, about nine copies), pIX (240 copies), or hexon (720 copies) proteins. b) Western blot analysis demonstrating the presence of 6-His tags in the Ad vectors in either FF (lane 2), pIX (lane 3), or hexon (lane 4). An unmodified Ad vector without a 6-His tag was used as a negative control (lane 1). c) AuNPs can be noncovalently coupled to specific locations on the Ad capsid. It is hypothesized that the Ni-NTA attached to the surface of 1.8-nm AuNPs will react with the Ad capsid proteins that display a 6-His tag, thus resulting in a specific high-affinity binding of AuNPs to Ad particles. The schematic diagram shows Ad vectors expressing a 6-His tag in the hexon protein. There are 720 potential sites of Ni-NTA-AuNPs coupling to the Ad corresponding to the number of hexon molecules present in the Ad capsid.

2. Results

2.1. Ad Vectors Utilized for NP Coupling

To demonstrate the feasibility of specific NP coupling to an Ad vector platform, we used AuNPs as representative examples.

To specifically couple NPs to Ad vectors, we exploited the noncovalent affinity of nickel(II) nitrilotriacetic acid (Ni-NTA) for a sequence of six-histidine amino acid residues (6-His). In particular, the Ni-NTA group on the AuNPs employed has a high affinity for a 6-His tag that can be genetically engineered into the Ad capsid at various defined capsid surface locations. We therefore utilized Ad vectors expressing 6-His tags in capsid proteins not essential for Ad infectivity. These include an artificial fiber called "fiber fibritin" (FF; about nine copies), Ad capsid protein IX (pIX; 240 copies), or hexon (720 copies, Figure 1a). First, the expression of a 6-His tag in the Ad capsid was verified by Western blot analysis of purified virions. When stained with an antibody recognizing 6-His, a band with the appropriate size was observed for all the viruses tested, with the expected size of FF, pIX, and hexon being 37, 14.4, and 109 kD, respectively (Figure 1b). In addition, the relative intensities of the bands depended on the copy number of a particular protein in the Ad capsid. For example, the band intensity of FF (Figure 1b, lane 2, about nine copies) is less than that of pIX (Figure 1b, lane 3, 240 copies), which in turn is less than that of hexon (Figure 1b, lane 4, 720 copies).

2.2. Specific Coupling of NPs to Ad Vectors

To couple AuNPs specifically to Ad vectors, Ni-NTA-AuNPs were reacted with the described Ad vectors that display 6-His tags at specific capsid locales (Figure 1c). For this purpose, an AuNP:Ad (particle:particle) ratio of 2000:1 was employed in the reaction mixture. After the reaction, the complexes were purified from unreacted AuNPs and Ad by using CsCl density-gradient ultracentrifugation-a standard method for Ad vector purification. After centrifugation of AuNP-labeled viral particles in a CsCl gradient, a shift in the viral band position (density) relative to that of unlabeled virus in the gradient was observed. The extent of the band shift in the centrifuge tube was dependent on the type of modified Ad vector used in the coupling procedure.

For a negative control we incubated Ni-NTA-labeled AuNPs with an Ad vector that did not contain a 6-His tag. The resulting Ad band had the same density in the CsCl gradient as the same unmodified Ad without incubation with Ni-NTA-AuNPs (Figure 2a; Table 1 in the Supporting Information). This finding indicates that Ni-NTA-AuNPs, as expected, did not bind to Ad vectors in the absence of 6-His

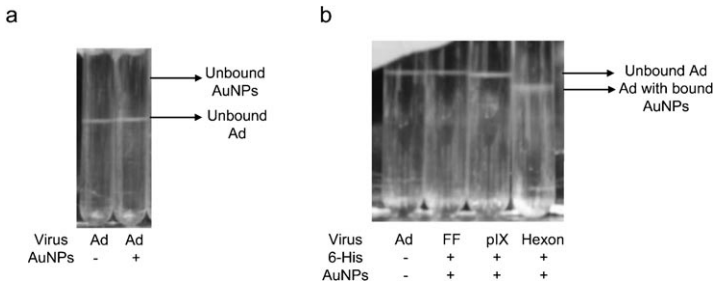

Figure 2. NP-labeled Ad vectors demonstrate a change in density in CsCl gradients. a) Ni-NTA-AuNPs do not bind to Ad vectors in the absence of 6-His tag, as revealed by density analysis in the CsCl gradient. Equal numbers of unmodified Ad particles (10^{12} viral particles) were analyzed by CsCl gradient centrifugation either without AuNPs (left tube) or following incubation with AuNPs at an Ad ratio of 2000:1 (AuNPs:Ad, right tube). The unbound AuNPs in the right-hand tube remain in the upper part of the gradient and form a diffuse zone. The sharp white band of material seen at the same position in both tubes contains uncoupled Ad vectors. b) Relative positioning of the AuNP-coupled and uncoupled Ad vectors in the CsCl density gradient. Equal numbers of Ad particles (10^{12} viral particles) without or with a 6-His tag in FF, pIX, or hexon were incubated either with Ni-NTA-AuNPs at the AuNP:Ad input ratio of 2000 (three tubes on the right) or no AuNPs (tube on the left). The change in the viral band density seen for the Ad vector expressing a 6-His tag in hexon (far-right tube) indicates an efficient coupling of the AuNPs to Ad.

sites. Unexpectedly, when Ni-NTA-AuNPs were incubated with the Ad vectors containing a 6-His tag in either FF or pIX, no change in the virus density, that is, band shift was observed (Figure 2b; Table 1 in the Supporting Information).

In contrast to the control and Ad vectors containing 6-His tag in FF and pIX, a distinct change in the viral band density upon AuNP coupling was observed for the Ad vector containing a 6-His tag in hexon (Figure 2b; Table 1 in the Supporting Information). Hexon has four loop regions (L1 to L4), three of which are located on the outside of the virion being exposed to a solvent. Within these loops there are nine hypervariable regions (HVRs) with no known function.[9] Two of these, HVR2 and HVR5, can display heterologous peptides that are accessible for binding and retargeting of the mature virion.[10] In this study we used a modified Ad with a 6-His tag incorporated in the HVR2 for coupling to AuNPs. The result shown in Figure 2b confirms the accessibility of this location for the interaction with Ni-NTA-AuNPs. To further prove the specificity of the Ni-NTA-AuNP interaction with Ad vector expressing 6-His in HVR2, this reaction was also performed in the presence of imidazole (250 mM), which, as expected, competitively inhibited AuNP binding to the Ad vector (Figure 7 in Supporting Information). Thus, based on the buoyant density of the treated Ad vectors, it appears that the likelihood of NP coupling to Ad correlates with the number and potential accessibility of the available binding sites on the Ad capsid.

2.3. Transmission Electron Microscopy of NP-Labeled Ad Vectors

To confirm binding of NPs to the Ad particles, we used transmission electron microscopy (TEM) to visualize AuNPs on the surface of Ad particles that were purified by CsCl centrifugation, as described above. As expected, no AuNPs could be detected on either unlabeled Ad vector alone (Figure 3a) or Ad vector without 6-His but treated with AuNPs (Figure 3b). In line with our observations from the CsCl density analysis, no AuNPs were seen in the preparations of Ad vectors containing a 6-His tag in either FF or pIX (Figure 3c and d, respectively). In contrast, AuNPs could clearly be observed in the Ad vector containing a 6-His tag in hexon (Figure 3e and f). This result reinforces our conclusion that the selective coupling of NPs to the Ad vectors depends on the number and accessibility of the coupling sites on the Ad capsid surface, and identifies hexon as an optimal location for such coupling.

2.4. Atomic-Absorption Spectroscopy of NP-Labeled Ad Vectors

To further characterize the AuNP-labeled Ad vectors, atomic-absorption spectroscopy was used for quantification of the AuNPs coupled to the Ad vectors. The Ad vector with no coupled AuNPs and the one containing a 6-His tag in hexon, which showed both the change in viral band density and the presence of AuNPs by TEM upon coupling to Ni-NTA-AuNPs, were subjected to spectroscopy. The analysis demonstrated that on average this vector bound 56 ± 4 AuNPs (see Supporting Information), whereas the control Ad vector bound none. This validates the observation that hexon is a good capsid location for coupling NPs to Ad vectors.

2.5. Selectively NP-Labeled Ad Vectors Retain Infectivity in HeLa Cells

In our previous report■citation please■, we observed a drastic abrogation of native Ad infectivity upon nonspecific AuNP coupling at high AuNP:Ad ratios, possibly due to modification of Ad capsid proteins, such as fiber and penton base. To circumvent this problem, we envisaged specific coupling of AuNPs to the Ad capsid at locations not implicated in the natural mechanism of Ad infection. As described above, we were able to couple AuNPs specifically to hexon protein, which represents one of the structural proteins of the Ad capsid.

To determine whether our hypothesis was correct, we analyzed transgene expression in cells infected with Ad vec-

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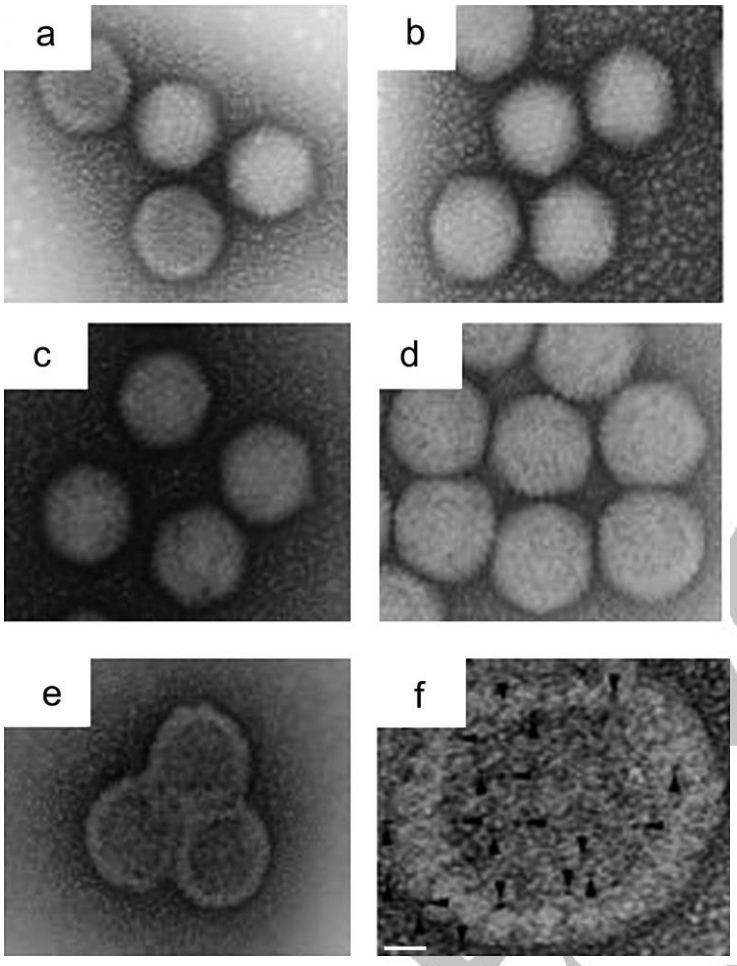

Figure 3. Visualization of AuNPs coupled to Ad vectors by TEM. Vectors were either unlabeled (a) or labeled with Ni-NTA-AuNPs to 6-His tag expressed in either FF (c), pIX (d), or hexon (e). AuNPs coupled to Ad vectors are only observed for the Ad vector with a 6-His tag in hexon (e; magnified in (f) with black arrowheads pointing at 1.8-nm AuNPs). The Ad vectors that do not express a 6-His tag do not bind any AuNPs (b). Original magnification: 100 000×; scale bar: 10 nm.

tors encoding luciferase, with or without AuNPs coupled to hexon. To this end, we utilized HeLa cells, which have been previously reported to be readily susceptible to infection with Ad vectors. We observed a statistically significant, but only moderate, decrease in viral infectivity in the presence of bound AuNPs, as compared to the control where no AuNPs were present (Figure 4a). Despite this moderate loss of infectivity, the specifically AuNP-labeled Ad vectors (2000 AuNPs per Ad vector input ratio) retain their capability of infecting HeLa cells to a greater extent than the previously reported Ad vectors with nonspecifically coupled AuNPs, where addition of 1000 AuNPs per Ad vector to the reaction mixture resulted in a decrease of approximately one order of magnitude, and 3000 AuNPs per Ad vector resulted in a decrease of more than two orders of magnitude.[8] Thus, specific NP coupling to Ad vectors perturbs Ad infectivity to a lesser extent than the nonspecific NP coupling reported previously.

2.6. Selectively NP-Labeled Ad Vectors Can Be Retargeted to CEA-Expressing Tumor Cells

The efficiency and specificity of transduction of the Ad vectors to be used as a delivery platform for NPs predicates the efficacy of the NP:Ad complex targeting of specific cells. However, the majority of target cells, including tumor, endothelial, and dendritic cells, are defi-

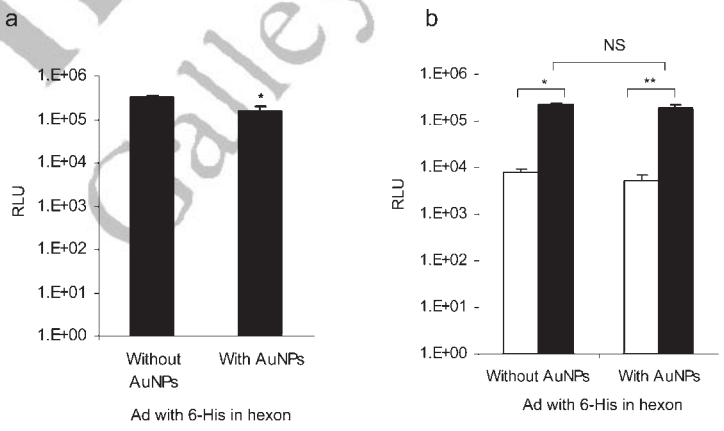

Figure 4. Analysis of infectivity and targeting of NP-labeled Ad vectors. a) The infectivity of Ad vectors is moderately affected upon specific AuNP coupling. A moderate reduction in the infectivity of AuNP-labeled Ad vectors in HeLa cells was observed as compared to the unlabeled vectors ($n=3$, $^{*}p=5.8\times10^{-3}$). b) The retargeting efficiency of the AuNP-coupled Ad vector to CEA-expressing cells remains unaffected by the site-specific gold coupling of the virus. The amount of luciferase transgene delivered to MC38-CEA-2 was similar for both unlabeled and AuNP-labeled Ad vectors in the presence of sCAR-MFE fusion protein, which retargets the viral vector to the expressed CEA ($n=3$, NS: not significant, $p=1.054\times10^{-1}$). For both unlabeled and AuNP-labeled Ad vectors, the amount of luciferase transgene delivered is significantly more in the presence of sCAR-MFE fusion protein ($n=3$, $^{*}p=4.482\times10^{-6}$, $^{**}p=1\times10^{-4}$). Bars represent mean values ± standard deviation. ■Please define RLU■

cient in the primary Ad receptor, the coxsackie adenovirus receptor (CAR), thereby resulting in poor tumor-cell transduction. To improve the transduction efficiency, a variety of approaches have been developed whereby the Ad vector is physically retargeted to alternate receptors on the target cell surface.[11] An example is retargeting of Ad vectors to the tumor-associated antigen carcinoembryonic antigen (CEA), which is overexpressed on several neoplasias, such as colon and breast carcinomas. We used a bifunctional adapter molecule, sCAR-MFE, for retargeting Ad vectors to CEA-expressing tumor cells. The sCAR-MFE protein binds to the fiber knob in the Ad capsid through the sCAR part of the molecule and to the CEA on tumor cells through the MFE part of the molecule, which is a single-chain antibody (MFE-23) directed to CEA.

In our previous report■reference?■, we observed detrimental effects on Ad vector retargeting to CEA-expressing tumor cells upon nonspecific NP coupling at high NP:Ad ratios. To determine whether specific NP coupling to Ad vectors could reduce the negative effects on Ad retargeting to CEA, Ad vectors coupled and not coupled to AuNPs through the 6-His tag modification in hexon were preincubated with the bifunctional adapter molecule sCAR-MFE. To analyze the targeting efficiency of AuNP-coupled Ad vectors complexed with sCAR-MFE, we utilized the CEA-expressing cell line MC38-CEA-2. For various coupling reactions, we used an Ad:AuNP input ratio of 1:2000 and the purified Ad:AuNP complex was incubated with sCAR-MFE in a ratio of 1:85 000 before addition to the cells. We used an excess of AuNPs and sCAR-MFE molecules to saturate all potential binding sites on the Ad vector. Notably, the amount of sCAR-MFE used does not saturate the CEA receptors on the target cells.[12]

In contrast to the previously reported decrease in targeted gene transfer using a nonspecific NP coupling approach, we observed no statistically significant difference ($p > 0.05$) in the targeted gene transfer for the vectors and containing a 6-His tag in hexon, with and without the prior AuNP coupling step. In addition, we observed a significant increase ($p < 0.01$) in the level of gene transfer of both AuNP-coupled and noncoupled Ad in the presence of sCAR-MFE as compared to the same viruses that were not preincubated with this targeting adapter molecule (Figure 4b). Thus, in contrast to the previously reported *nonspecific* coupling strategy, the *specific* NP coupling to Ad capsid has no detrimental effect on Ad retargeting.

3. Discussion

Nanotechnology presents novel opportunities for the imaging and treatment of diseases, such as the use of QDs for visualizing disease processes or gold nanoshells for photothermal therapy of cancer. In this regard, combination of nanotechnology with gene therapy would result in multifunctional nanoscale systems with potential for sophisticated disease treatment, for example, in the context of cancer and cardiovascular disease. Towards this end, we have developed a methodology for specifically coupling NPs to an Ad vector, which is a well-developed human gene therapeutic vector currently in many clinical trials. Importantly, the coupling method does not negatively affect virus infectivity and targeting to specific cells. However, we would like to point out that the observed unperturbed gene-transfer ability is only indirect evidence for the cellular uptake of AuNP-labeled Ad vectors; technical difficulties with observing 1.8-nm AuNPs in a cellular environment prevent the observation of AuNP-labeled Ad vectors inside an infected cell at this point in time. The coupling of larger NPs may resolve this problem in future experiments.

For nanotechnology to become relevant as a treatment option, it is critical to achieve targeted delivery of NPs to specific cells. With regard to NP delivery, NPs have previously been targeted by utilizing both passive and active systems. Passive targeting approaches exploit the enhanced permeability and retention of "leaky" tumor vasculature, as demonstrated for Au nanoshells.[6] Although passive targeting showed promising results, active targeting to a tumor-associated antigen (TAA) is thought to be more effective. For this purpose, antibodies and peptides targeted against TAAs have been used to deliver AuNPs specifically to tumor cells.[13–16] However, there is a limit on the number of NPs that can be attached to either an antibody or a peptide, due to their small size as compared to liposomes and Ad vectors. To circumvent this problem, targeted liposomes can be used, in which NPs are encapsulated by a lipid bilayer that has targeting ligands immobilized on its outer surface.[17,18] However, if a combination of nanotechnology with gene therapy is desired, effective delivery of a therapeutic transgene to the target cells is critical. In this regard, Ad-vector-mediated gene transfer is still unparalleled in in vivo systems, vis-à-vis targeting potential and transduction efficiency, although much progress has been achieved with other vector systems in recent years. Thus, a viral vector platform would be optimal for the assembly and targeted delivery of NPs to specific cells resulting in a combination of gene therapy and nanotechnology for the treatment of disease.

To serve as a platform for NPs, a suitable method is required for coupling NPs to the viral vector. Towards this end, NPs have been coupled to viral capsids by utilizing nonspecific as well as specific chemistries. For instance, we have previously nonspecifically coupled AuNPs to lysine residues in the capsid of an Ad vector. However, due to the nonspecific coupling methodology, at higher NP:Ad vector ratios an abrogation of Ad vector infectivity and targeting to specific cells was observed.[8] Thus, a method for specific coupling of NPs to viral vector platforms would be optimal. In this regard, Belcher's group has coupled NPs specifically to the outer coat of bacteriophage M13.[19,20] However, phages cannot be used for human gene therapy applications, especially with respect to the envisioned combinatorial nanoscale multifunctional system. Herein, we therefore describe a specific coupling chemistry for human Ad vectors that does not negatively affect the retargeting ability of the virus, thus making it feasible to combine nanotechnology and gene therapy in one nanoscale system. Furthermore, the methodology described to specifically couple NPs to Ad vectors can be readily adapted for other gene therapy vec-

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tors, such as adeno-associated virus (AAV), herpes simplex virus, or lentivirus.

The methodology described herein utilizes the affinity of 6-His for Ni-NTA. This affinity is routinely used to purify recombinant proteins containing a 6-His sequence in laboratories across the world. Moreover, 6-His is nonimmunogenic and does not perturb the mature viral assembly. Also, it has been shown previously that a 6-His expressing recombinant Ad12 knob protein binds to Ni-NTA-labeled gold. In addition, this AuNP-labeled Ad12 knob protein is still bound to purified CAR protein.[21] However, Ad12 is not used for clinical gene therapy due to its oncogenic potential.[22] We therefore used the Ad5 vector, which has been used in multiple gene therapy trials. Although incorporation of a 6-His tag is possible in the Ad5 knob similar to the Ad12 knob, we chose not to use this AuNP-binding locale because of potential interference with targeting strategies, in which an adaptor molecule has to bind to the Ad knob and retarget the virus to an antigen expressed on a tumor cell. Based on this, we utilized Ad vectors expressing a 6-His tag in capsid proteins that are not involved in the Ad vector infectivity and targeting pathway, including an artificial fiber (FF), pIX, and hexon protein.

The absence of observed AuNP binding to the Ad vectors expressing a 6-His tag in FF, as evidenced by unchanged viral band density in the CsCl density gradient, might be explained by the fact that there are only a few FF copies (about nine) with a 6-His tag available for AuNP binding. We also did not observe any AuNPs bound to the FF protein in the Ad capsid in TEM. However, it must be realized that due to the flexibility of the Ad fibers it is extremely difficult to visualize these in TEM. We therefore do not completely disregard the notion that AuNPs can be coupled to FF, but judging from the lack of change in density the number of AuNPs per virion will be much less than hexon, should any bind at all.

With regard to the Ad vector containing a 6-His tag in pIX, the pIX protein has been located 65 Å below the surface of the Ad capsid in a cavity between hexon proteins,[23] and thus the 6-His moiety is likely to be inaccessible for AuNP coupling. In addition, the C-terminal domain of the pIX protein has been proposed to exist in two different conformations, either bound to the capsid or extended away from the capsid.[24] Thus, the flexibility in the pIX protein conformation might hinder the binding of Ni-NTA-AuNPs to a 6-His tag expressed in pIX.

With regard to the hexon coupling location, it is noteworthy that with an Ad:AuNP input ratio of 1:2000 and 720 copies of hexon per virus, only 56 AuNPs bound per virion. This leads us to speculate that steric hindrance might play a role in AuNP interaction with the hexon protein in the Ad capsid. One potential reason may be the location of HVR2, where our 6-His tag is inserted, partially underneath the larger HVR1 region.[25] This partial obscuring of HVR2 may result in a suboptimal coupling efficiency of AuNPs. In addition, the spatial location of various Ad capsid proteins is still an area of active research, as evidenced by a recently published study by Marsh et al.[26] Based on their results, they reassigned the position earlier thought to be occupied by the protein IIIa to the pIX protein. The capsid proteins surrounding the hexon protein might therefore impose additional steric constraints. As the details of the Ad capsid structure are determined with greater accuracy, the issues pertaining to the steric hindrance to interaction of various capsid proteins with molecules like AuNPs will be better understood.

The specificity of the 6-His–Ni-NTA coupling is demonstrated by our results in which Ni-NTA-labeled NPs bound to Ad vectors only at those accessible capsid locations that expressed a 6-His tag in high numbers, as well as the ability of the imidazole to prevent the coupling reaction. Another interaction that could be utilized instead of 6-His–Ni-NTA is that of biotin with (strept)avidin. Viral vectors, such as Ad,[27] AAV,[28] and lentivirus,[29] which are metabolically biotinylated during virus production in specific capsid locations including hexon, have already been constructed by other groups. These viral vectors could be coupled to NPs with surface-attached (strept)avidin or coupled to biotinylated NPs via a (strept)avidin bridge.

The specific AuNP coupling to Ad vectors demonstrated here can be exploited for coupling of other types of NPs to gene therapy vectors. For example, magnetic NPs can be utilized for either magnetic-resonance-based imaging or magnetic-field-mediated tumor-cell hyperthermic ablation.[5] Another example would be the use of QDs, which have excellent imaging applicability. With regard to imaging, QDs are superior to traditional fluorescent labels owing to their consistent and prolonged signal strength.[30] Not only would QD-labeled Ad vectors be of use for imaging tumors, they would also be a sophisticated tool to track Ad vector biodistribution in preclinical animal models, thus illustrating the versatility of this approach.

In the aggregate, the specific coupling methodology realized herein for attaching NPs to Ad vectors provides an opportunity for specific assembly and delivery of NPs to target cells. In addition, the specific labeling of Ad vectors by NPs achieved in this study represents a unique combination of gene therapy and nanotechnology approaches, which has the potential for simultaneous targeting, imaging, and therapy of disease. This multifunctional nanoscale system, which is capable of incorporating multiple modalities in a single particle, provides an important basis for the development of new-generation diagnostics and therapies.

4. Conclusions

For successful utilization of the various treatment options offered by nanotechnology, target-cell-specific delivery of NPs is crucial. We have demonstrated that NPs can be specifically coupled to distinct Ad capsid proteins and targeted to tumor cells. In addition, specific NP-labeled Ad vectors displayed the same level of infectivity and targeting capability to tumor cells as unlabeled Ad vectors. Thus, Ad vectors can serve as the platform for selective self-assembly and targeted delivery of NPs to target cells. This paves the way for realization of a multifunctional nanoscale device for

the treatment of disease by combining gene therapy and nanotechnology approaches.

5. Experimental Section

Ad vector production: The Ad vectors encoding a firefly luciferase (Luc) and/or green fluorescent protein (GFP) under transcriptional control of the constitutively active cytomegalovirus (CMV) promoter, and displaying a 6-His tag in FF[31] and hexon (HVR2),[10] were constructed as described previously. The Ad vector displaying a 6-His tag on pIX was generated as follows. First, we constructed a modified pShuttle CMV vector[32] encoding Ad capsid pIX fused to a short linker peptide $P(SA)_4$-PGSRGS, followed by a 6-His tag downstream of the pIX-Flag open reading frame. The 15 amino acid linkers with 6-His were amplified by the polymerase chain reaction from pBS.F5.RGS6HSL[33] and cloned into the shuttle vector plasmid pSlLucIXflag[32] at the unique NheI site downstream of the pIX-flag coding sequence. Recombinant Ad was generated by homologous recombination with the adenoviral genome plasmid pAdEasy1 (Qbiogene, Carlsbad, CA) in *Escherichia coli* strain BJ5183, and virus was rescued in HEK-293 cells. For subsequent virus production, cells were infected using growth medium as described below, except containing 2% fetal bovine serum instead of 10%. Following overnight incubation, regular medium was added to the cells and they were incubated until a cytopathic effect was observed. The cells were harvested and lysates obtained by four consecutive freeze–thaw cycles. Virus was purified by standard double CsCl gradient centrifugation. The viral particle number was determined by measuring the absorbance at 260 nm using a conversion factor of 1.1×10^{12} viral particles per absorbance unit.[34]

Cell culture: HEK-293 cells were obtained from Microbix (Toronto, Canada), HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and MC38 cells stably transfected with CEA, MC38-CEA-2, were kindly provided by Dr. Jeffrey Schlom, National Cancer Institute (Bethesda, MD). All cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1 v/v, Mediatech, Herndon, VA), which contained fetal bovine serum (10%, Hyclone, Logan, UT), L-glutamine (2 mM), penicillin (100 IU mL^{-1}), and streptomycin (25 μg mL^{-1}; all Mediatech). Medium for MC38-CEA-2 cells additionally contained G418 (500 μg mL^{-1}, Mediatech). Cells were grown in a humidified atmosphere with CO_2 (5%) at 37°C.

Western blotting: A total of 10^9 viral particles of each viral vector were mixed with Laemmli sample buffer containing β-mercaptoethanol (10 mM). Samples were boiled for 10 min at 95°C and were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 4–15% polyacrylamide gradient) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membranes were probed with an anti-His tag monoclonal antibody (Molecular Probes, Invitrogen, Eugene, OR). This was followed by a horseradish peroxidase conjugated goat antimouse secondary antibody (DakoCytomation, Carpinteria, CA). Signals were detected using Western Lightning chemiluminescence reagent (Perkin–Elmer Life Sciences, Boston, MA) and Kodak BioMax MR Film (Kodak, Rochester, NY).

Coupling of AuNPs to the Ad vector: AuNPs 1.8 nm in size and containing a Ni-NTA reactive group on the surface of the particle were acquired from Nanoprobes (Yaphank, NY). As per the information received from Nanoprobes, elemental analysis for nickel and gold suggests an average of between 15 and 20 NTA-Ni(II) per Nanogold. Also, the compound is supplied as the Ni(II) chelate. The reaction of AuNPs with Ad vectors was carried out at a ratio of 1:2000 (Ad:AuNPs) in a buffer of pH 7.5 containing Tris (20 mM) and NaCl (15 mM) for 30 min at room temperature, with 10^{12} viral particles of Ad used for each reaction. The reaction mixture was subsequently loaded onto a CsCl density gradient.

Purification of AuNP-labeled Ad vectors: To determine whether AuNPs were coupled to the Ad vectors, reaction mixtures were purified using a CsCl density gradient with ultracentrifugation at 25 000 rpm for 3 h at 4°C. Following ultracentrifugation, the distance of viral bands was measured from the bottom of the tube (Table 1, Supporting Information). For further experiments, viral bands were collected from the bottom of the centrifuge tube.

Electron microscopy of AuNP-labeled Ad vectors: Unmodified or AuNP-labeled Ad vectors were deposited onto carbon-coated copper grids, washed with double-distilled water, stained for 10 s with Nano-Van (Nanoprobes, Yaphank, NY), and examined using a JEOL JEM 1200FX electron microscope with a point resolution no worse than 0.45 nm. The microscope was operated at 80 kV and carefully aligned according to Nanoprobes' instructions to directly visualize the 1.8-nm gold particles. Negatives were recorded at 100 000 magnification and subsequently digitized with a Nikon SuperCoolScan scanner at 1800 dpi, which produced a pixel resolution of 0.14 nm in images of size 4033×6010 pixels.

Atomic-absorption spectroscopy of AuNP-labeled Ad vectors: The gold atomic-absorption standard solution (1 mg mL^{-1} in 0.5N HCl) was obtained from Acros Organics (Belgium) and diluted to make standards ranging from 10 to 100 ppb. The obtained Au atomic-absorption standard solutions were used for instrument calibration as well as for a quality-control measurement. The atomic-absorption measurements were performed at Atomspec DF Workstation (Thermo Jarrell Ash Corporation). The atomic absorption of Au was measured at 242.8 nm using the Smith–Hieftje background correction method. Before measuring the Ad:Au samples, they were dialyzed to remove CsCl and replace it with water. Then the viral particle number was determined as described earlier. The number of AuNPs per virion was calculated by comparing the atomic-absorption readings for the viral samples with the Au standard, assuming 180 atoms of Au per AuNP. For calculation details, please see the Supporting Information.

Construction, production, and purification of the retargeting adapter molecule sCAR-MFE: A fusion protein capable of retargeting Ad vectors to the tumor-associated antigen CEA, which consists of the ectodomain of CAR followed by a five amino acid peptide linker (GGPGS), a six-histidine tag (for detection/purification), and the anti-CEA single-chain antibody MFE-23 (a kind gift from Dr. Kerry Chester, London, UK), was constructed, produced, and purified as described previously.[8]

In vitro gene transfer: To assess Ad infectivity, HeLa cells were plated in triplicates at a density of 10^5 cells/well in 24-well plates. The following day, 10^7 viral particles of Ad vectors (100 viral particles per cell) were added to the cells in medium containing fetal bovine serum (2%). After 2 h of incubation, medium containing Ad vectors was removed and replaced with regular growth medium. Cells were incubated for an additional 22 h and were subsequently washed with phosphate-buffered saline and lysed using Reporter Lysis Buffer (Promega, Madison, WI). After one freeze–thaw cycle, luciferase activity was measured using the Luciferase Assay System (Promega), according to the manufacturer's instructions. To assess retargeting of Ad vectors to CEA by the sCAR-MFE fusion protein, MC38-CEA-2 cells were plated and infected as described above, with viral particles being incubated for 15 min at room temperature with 75 ng fusion protein (Ad:sCAR-MFE = 1:85 000) before addition to the cells.

Statistics: Statistical analysis for significance was performed using a two-tailed t-test assuming equal variance in Excel (Microsoft Office 2003).

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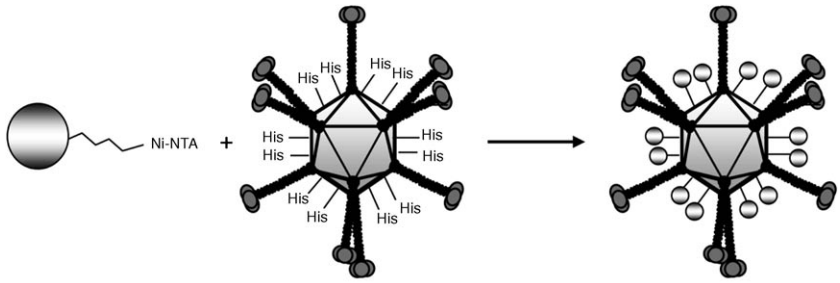

On target: Selective assembly of gold nanoparticles (AuNPs) on the surface of adenoviral (Ad) vectors allows a combination of gene therapy and nanotechnology for the treatment of diseases (see picture; Ni-NTA = nickel nitrilotriacetic acid). The specific coupling of NPs does not impede the ability of the virus to bind to target cells and deliver its transgene.

Viral vectors

V. Saini, D. V. Martyshkin, S. B. Mirov, A. Perez, G. Perkins, M. H. Ellisman, V. D. Towner, H. Wu, L. Pereboeva, A. Borovjagin, D. T. Curiel, M. Everts* _____ ■■■■ – ■■■■

An Adenoviral Platform for Selective Self-Assembly and Targeted Delivery of Nanoparticles

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Importance of Viruses and Cells in Cancer Gene Therapy

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ABSTRACT

Viruses have a documented history for being used in treatment and prevention of diseases for centuries, with their application in vaccination strategies as a prime early example. In more recent history, viral vectors have been employed for gene and cell therapy of tumors. In this regard, the increased understanding of the aberrant molecular pathways underlying the process of tumorigenesis has rationalized genetic correction of these pathophysiological processes using viral vector based gene and cell therapy approaches. For example, viruses have been genetically engineered to develop oncolytic potency or mediate long-term gene expression. Also, viral vectors carrying therapeutic genes or targeting molecules have been loaded into cells, which can be exploited as delivery vehicles for these therapeutic payloads to the desired target site. However, issues pertaining to viral and cell targeting as well as host immune response elicited upon viral or cell administration remain to be addressed. In summary, the plasticity of the viral structure has rendered them amenable for the development of unique gene and cell therapy approaches, for the treatment of tumors.

Keywords: cell vehicles, immune evasion, tumor-targeting, viral vectors
Abbreviations: Δ**24**, delta-24; **AAV**, adeno-associated virus; **Ad**, adenovirus; **Ad5/H3**, Ad3 hexon protein; **Ad5/H5**, Ad5 hexon protein; **APC**, antigen presenting cells; **AuNPs**, gold nanoparticles; **CAR**, coxsackie adenovirus receptor; **CEA**, carcino-embryonic antigen; **Cox-2**, cyclooxygenase-2; **CTL**, cytotoxic T lymphocytes; **E**, early; **EGF**, epidermal growth factor; **EGFR**, epidermal growth factor receptor; **flt-1**, vascular endothelial growth factor receptor Type-1; **GCV**, gancyclovir; **HSV**, herpes simplex virus; **HSV-1**, herpes simplex virus Type-1; **HVS**, herpesvirus Samiri; **IFN**, interferons; **IgG**, immunoglobulin G; **IL-12**, interleukin-12; **MHC**, major histocompatibility complex; **MV**, measles virus; **PEG**, poly(ethylene glycol); **PKR**, RNA-activated protein kinase; **RCA**, replication competent adeno-viruses; **RGD**, arginine-glycine-aspartate; **SCC**, squamous cell carcinoma; **scDb**, single chain diabody; **scFv**, single chain antibody; **TAM**, tumor-associated macrophages; **TCR**, T cell receptor; **TIL**, tumor-infiltrating lymphocytes; **TK**, thymidine kinase; **Tregs**, regulatory T cells; **VEGF**, vascular endothelial growth factor; **VSV**, vesicular stomatitis virus

CONTENTS

INTRODUCTION

Viruses have been utilized for therapeutic purposes for many centuries. They are interesting biological entities harboring on the borderline between non-living things and living organisms. Upon infection of the host cells, viruses manipulate the cellular machinery to their own advantage. This ability of viruses to induce changes in the target cells presented them as one of the most suitable candidates for serving as gene therapy vectors. A variety of viral vectors has been developed for gene therapy, such as herpes simplex virus (HSV), adenovirus (Ad), adeno-associated virus

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Invited Review

(AAV) and measles virus (MV), just to name a few. Although viral vector-based gene therapy has demonstrated great potential for treatment of diseases like cancer, many hurdles still need to be overcome before the full potential of viral vectors can be realized.

Cell therapy describes the implantation of cells to achieve a therapeutic purpose. This definition includes routine medical procedures, such as bone marrow transplants and blood transfusions, but it also encompasses the use of genetically manipulated cells for therapeutic purposes. Gene transfer, in general, can be used to replace a mutated gene in order to restore a natural cellular function, or to confer novel therapeutic modalities to a cell. Although viral vectors are efficient gene transfer agents, as described above, systemically administered virions can be nonspecifically sequestered or inactivated via innate or acquired immune mechanisms prior to reaching the intended target cell population. However, cells can be genetically loaded using viral vectors *ex vivo* and these transduced cells can then serve as vehicles to deliver the therapeutic payload to target sites *in vivo*. The combined use of gene and cell-based medicines allows for multifaceted approaches that may be required to treat complex diseases such as cancer.

The use of viruses for gene therapy is marred with problems such as targeted delivery of the viral vector to specific cells, the immune response against the vector and the resulting toxicity issues. Attempts to resolve these issues have resulted in the development of viral vectors with improved characteristics. In this review, we discuss the strategies that have been employed for the construction of viral vectors with enhanced potential for efficacious gene therapy. We outline the construction of 'gutless' and oncolytic viral vectors, which have improvements in terms of increased transgene carrying capacity and expression, improved therapy and enhanced safety. Following this, we discuss the various approaches that have been developed for targeting viral vectors to desired cell types, as well as strategies for host immune system evasion. We end with future considerations for the utilization of viral vectors for gene therapy.

VIRAL VECTORS AND THEIR MODIFICATIONS FOR GENE THERAPY

Many viruses have been used for gene therapy. However, multiple factors limit the effective utilization of viruses for gene therapy. For instance, it has been observed that upon transgene delivery to the target cells the transgene expression diminishes with time, warranting re-administration of the viral vectors. In this regard, viral vectors utilized for gene therapy can be either integrating or non-integrating. Integrating viruses, such as retroviruses (Chang *et al.* 2001) and AAV (McCarty *et al.* 2004), integrate their genome within the genome of the host organism. Non-integrating viruses, such as adenoviruses (Marini *et al.* 2002), do not integrate into the host genome, and therefore the viral genome is lost in proliferating cells. Historically it was therefore believed that integrating viral vectors would provide long-term expression of the therapeutic gene in the host and thus would not require repeated administration, unlike the non-integrating viruses. However, pre-clinical experience with the utilization of integrating vectors such as AAV for gene therapy has demonstrated that repeated administration might be necessary for integrating viruses as well. For example, when AAV was used for genetic correction of a cystic fibrosis defect in the lungs, the limited viral transduction efficiency resulted in low therapeutic gene delivery to the lung cells. Moreover, an antibody response generated against the viral vector reduced the amount of gene transfer that could be achieved and also prevented re-administration of the virus (Halbert *et al.* 2000). Modification of viral vectors to circumvent or mitigate an immune response against the infected cell and the vector itself is thus warranted, even if integrating vectors are used.

Gutless vectors

As noted above, administration of viral vectors results in an immune response. Upon first vector administration, the body responds by mounting an immune response against the virus itself, viral proteins that are expressed in the infected cells and the therapeutic gene if it is foreign to the host. This immune response severely limits the efficacy of the therapeutic vector since infected cells that express the transgene will be cleared from the body. In addition, the development of immunological memory restricts the efficacy of subsequent administrations, and limits the dosage and the number of times the viral vector can be administered to the patient. To circumvent the immune response generated against the viral vector and the viral proteins, one of the strategies employed is the deletion of the unnecessary viral genome sequences. The removal of the unnecessary viral genome sequences drastically reduces the immunogenicity of the viral vector, and increases the efficacy of viral gene therapy. Another benefit of the deletion of viral genome sequences is the increase in carrying capacity for foreign therapeutic genes. This is especially important when large genomic sequences need to be delivered, such as the dystrophin gene for the treatment of Duchenne muscular dystrophy (Bogdanovich *et al.* 2004).

As a representative example of viral vectors with deleted genome sequences, the construction of adenoviral (Ad) vectors carrying progressively less amounts of the viral genome can be studied, which is described below.

First generation Ad vectors

One of the considerations in deciding which viral genes can be deleted from the genome is the role played by these various genes in the viral reproduction cycle. As an example, for Ads it was discovered that early (E) expression gene products could be provided *in trans* in order to achieve mature adenoviral progeny production during the production process. In particular, E1, E2, E3 and E4 regions have been deleted or inactivated. Initially, it was the E1 region that was deleted from the Ad genome considering its essential role in transcriptional activation of other early genes, inhibition of apoptosis of the infected cell and modification of the intracellular environment to make it more conducive for Ad protein production (Akusjarvi 1993; Flint *et al.* 1997; Young *et al.* 1997; Dyson 1998). Deletion of E1 resulted in replication deficient viral vectors that were propagated in helper cell lines that provided E1 gene product *in trans* (Trapnell *et al.* 1994).

Subsequently, the E3 region was deleted, which encodes proteins that inhibit various death pathways elicited by the host immune system against the cells infected with Ad vectors (Wold *et al.* 1995, 1999). The Ad vectors with deleted E1, with or without deletion of E3, are referred to as 'first generation' Ad vectors (**Fig. 1**). The first generation Ad vectors have a carrying capacity of ~8 kb for foreign genes (Bett *et al.* 1993). However, unfortunately, even after deletion of E1 and E3, these viral vectors still suffer from immune resistance due to leaky viral protein expression in the host. This results in clearance of the viral vectors as well as host cells infected with the virus (Yang *et al.* 1994). In addition, propagation of these vectors in complementing cell lines may result in replication competent adenoviruses (RCA) due to recombination with the viral DNA sequences present in the complementing cell line (Amalfitano *et al.* 1998). The RCA contaminates the replication incompetent viral vector preparations. The possibility of uncontrolled replication of this RCA contaminant in the patient increases the safety considerations.

Second generation Ad vectors

The problems with the first generation Ads mentioned above sparked the further minimalization of the viral genome, and thus the viral protein expression in the host. For

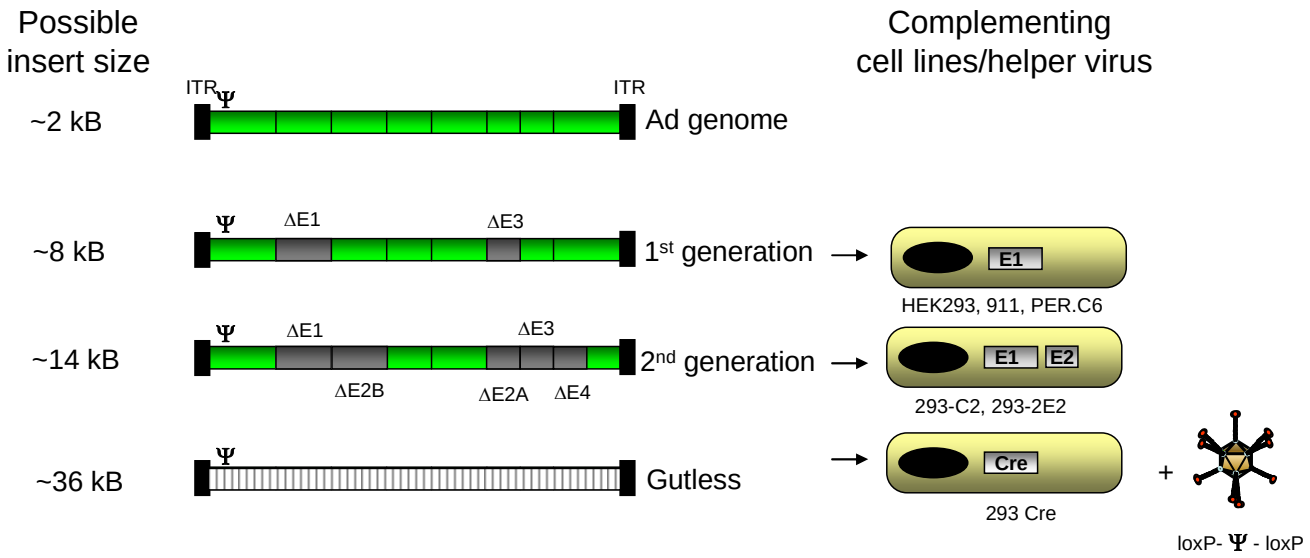

Fig. 1 Diagram of viral genomes corresponding to the wild type Ad genome and three generations of Ad vectors. Deleted genes are shown in gray. The function of the deleted genes is delivered *in trans* by complementing cell lines or a helper virus. Each generation has tolerated larger insert sizes, culminating in gutless vectors that can package inserts up to 36kB. An example of how these gutless vectors are produced is the use of a helper virus that incorporates loxP sites that flank the packaging signal (ψ) in its genome. When this virus infects cells that express the Cre recombinase and are transfected with the gutless genome, the packaging signal will be deleted from the helper virus genome that will thus not be incorporated into the new virions, resulting instead in packaging of the gutless genome that does have the packaging signal.

this, in addition to E1 and E3, the E2 region was also deleted (Amalfitano et al. 1998). The E2 region encodes proteins needed for Ad DNA replication (van der Vliet 1995). Following the E2 deletion, the E4 region was also deleted. The E4 region encodes multiple proteins that are utilized for Ad DNA replication, mRNA transport and splicing, inhibition of host cell protein synthesis, and regulation of apoptosis (Bridge *et al.* 1989; Huang *et al.* 1989). With regards to E4, viral vectors with modifications other than deletion, such as removal of the E4 promoter, have also been generated. The vectors with deletions in E2 and E4, along with E1 and E3 in different combinations, are referred to as 'second generation' Ad vectors (**Fig. 1**). This second generation has a transgene carrying capacity of ~14 kb (Alba *et al.* 2005).

In addition to reducing the host immune response and increasing the transgene carrying capacity of Ad vectors, these deletions also resulted in more severely crippled replication deficient vectors than the first generation vectors, thereby increasing their safety profile (Parks *et al.* 1996). For example, an Ad vector carrying the tumor suppressor p53 in the deleted E1 region, deleted for E3 and having an inactivated E4 region was compared to a vector with a wild type E4 region, to analyze whether deleting multiple viral genes can enhance the safety profile of the Ad vector. The Ad vector with the inactivated E4 region demonstrated a reduced host immune response compared to the control vector, resulting in reduced toxicity and prolonged duration of p53 expression *in vivo* in immunocompetent mice (Ji *et al.* 1999).

However, despite these encouraging results, the residual gene expression from the remaining viral genes still resulted in immunogenicity and toxicity for these second generation Ad vectors. In this regard, it was soon realized that for the Ad vectors, in addition to the early region genes, many more genes could be deleted and their functions provided *in trans*. Thus, true "gutless" vectors came into being.

Third generation 'gutless' Ad vectors

Gutless vectors are the most advanced form of Ad vectors currently available. These vectors are devoid of all the viral genes except those that are required *in cis* for packaging and replication. These vectors are also known as gutted, amplicon, high-capacity, helper-dependent and fully-deleted adenoviral vectors (**Fig. 1**). The transgene carrying capacity of gutless vectors is ~36 kb (Alba *et al.* 2005). These vectors have demonstrated a better safety profile than the first and second generation of Ad vectors. However, there are still some problems with gutless Ad, especially in regard to problematic production of high titers that are required for clinical use. Also, contamination with RCA remains a concern that requires further investigation (Alba *et al.* 2005). These problems are currently being countered utilizing various approaches, such as episomally maintained Ad vectors (Kreppel *et al.* 2004) and improved packaging cell lines (Sakhuja *et al.* 2003; Alba *et al.* 2005).

In addition to the above mentioned 'gutless' Ad vectors, other viral vectors with deleted viral genomes have been constructed. For example, lentiviral (Naldini *et al.* 2000) and retroviral vectors devoid of viral genome sequences in the transfer vector have been constructed, such that no viral proteins are produced in the infected cells.

In conclusion, even though many issues pertaining to efficient production of the gutless vectors still need to be resolved, it is anticipated that gutless vectors will be increasingly used for gene therapy in coming years due to their improved efficacy and safety profile.

Oncolytic viral vectors

The proposed use of viruses for gene therapy applications has always caused concern because of the inherent pathogenic nature of these agents. In this regard, viral vectors were modified to limit their replication potential in the host organism (**Fig. 2**). Therefore, initially only the gene delivery capacity of viral vectors was utilized for gene therapy. Although this addressed the concerns related to safety issues in a cancer therapy context, this also prevented the use of a very efficient cell killing method, i.e., viral vector mediated lysis of infected tumor cells. For example, replication deficient Ad vectors were utilized to deliver a bacterial cytosine deaminase gene into glioma cells, which chemosensitizes glioma cells for otherwise non-toxic 5-fluorocytosine (Dong *et al.* 1996). This strategy kills those tumor cells which are infected with the viral vectors, but not the remaining tumor cells. However, if the viral vector could replicate selectively in the tumor cells thereby resulting in oncolysis, then the viral progeny could potentially infect

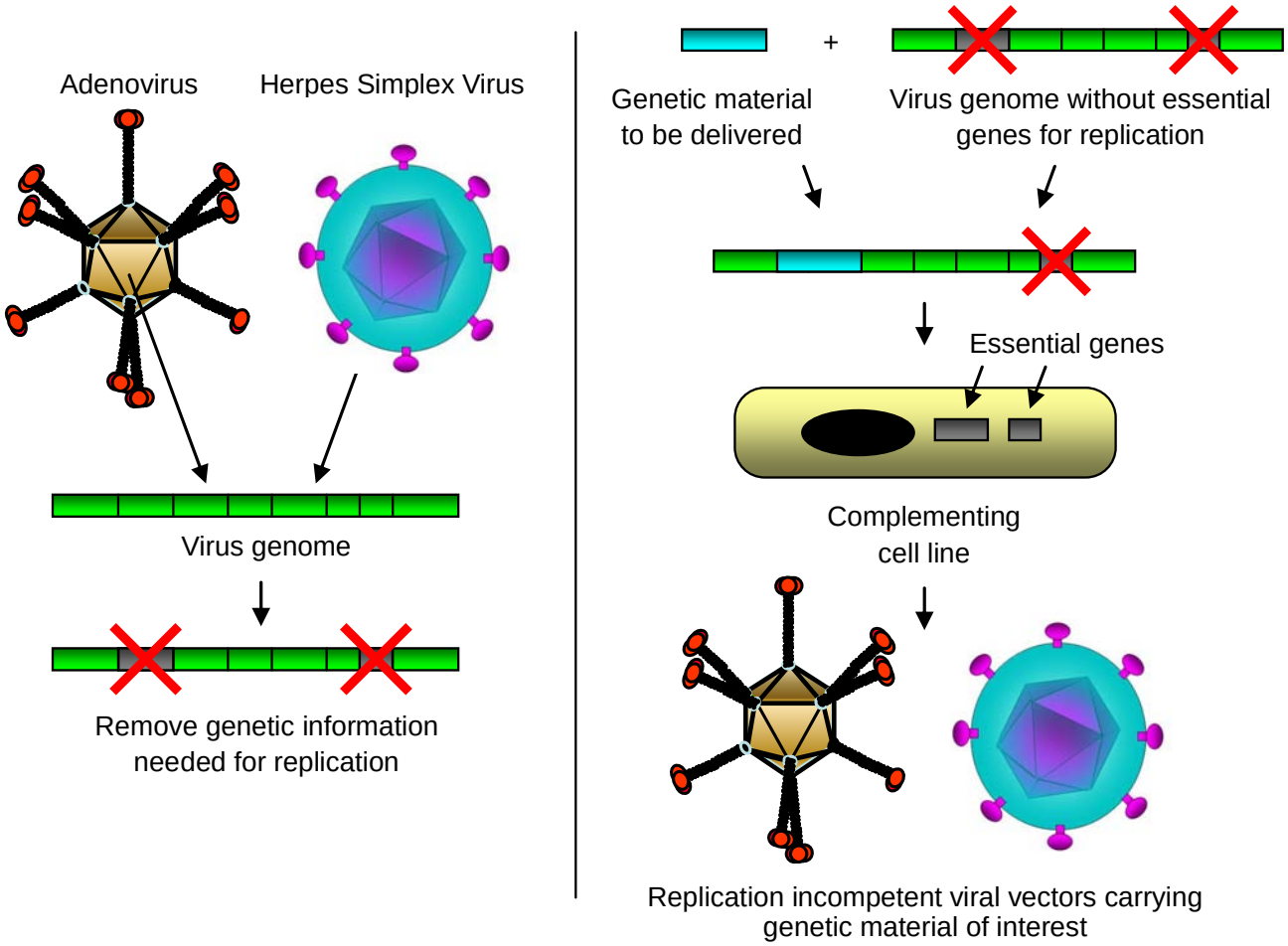

Fig. 2 Modification of replicating viruses into non-replicating gene therapy vectors. Left: Adenovirus and Herpes Simplex Virus are examples of viruses that can be modified into replication incompetent gene therapy vectors by deleting the genes necessary for viral replication (gray rectangles) from the viral genome (green rectangles). Right: The deletion of genes essential for viral replication provides space for therapeutic genes of interest (blue rectangle), which can be incorporated into the genome. For vector production, the gene products necessary for viral replication (gray rectangles) are provided *in trans* in a complementing cell line, resulting in replication incompetent vectors that carry the therapeutic gene of interest.

the adjoining tumor mass that escaped the primary infection. Moreover, replicative virus can kill tumors in combination with the chemosensitizing approach.

Thus, in order to utilize the inherent cell killing potential of viruses with a lytic replication cycle but avoid side-effects in healthy cells, viral vectors capable of selective replication in tumor cells were constructed. These viral vectors are replication competent and thus oncolytic, but only in target cells by using a variety of mechanisms, as will be described below. The use of oncolytic viruses for killing target tumor cells has been defined as virotherapy (Nettelbeck *et al.* 2003).

Advantages of oncolytic viral vectors

There are multiple advantages that mandate the use of conditionally replicative oncolytic viruses for tumor treatment. Being replicative, after the initial infection, viral progeny can spread through the tumor mass and effectively remove all of the tumor cells. In addition to their oncolytic properties, these viruses can also introduce therapeutic genes, such as suicide genes and cytokines. In addition, expression of viral proteins can be utilized to elicit an anti-tumor immune response, increasing the effectiveness of tumor treatment.

A variety of oncolytic viruses have been used as potential candidates for oncolytic therapy, including Herpes Simplex Virus (HSV), reovirus, vesicular stomatitis virus (VSV), and Ad, to name a few.

The viruses currently under investigation for oncolytic therapy are either inherently selective or are genetically modified to be selective for replication competence in tumor cells. In this regard, herpesvirus samiri (HVS) was demonstrated to be naturally selectively oncolytic for the pancreatic cancer line PANC-1 (Stevenson *et al.* 2000). Similarly, human reovirus (Hashiro *et al.* 1977) and VSV (Stojdl *et al.* 2000) were shown to replicate more efficiently in transformed cell lines as compared to non-transformed cells lines (Ring 2002). Reovirus is an example of a naturally oncolytic virus with replication limited to tumor cells with an activated Ras-signaling pathway. Upon infection of normal cells by reovirus, the early viral transcripts activate double-stranded RNA-activated protein kinase (PKR), which inhibits viral protein translation and viral replication. However, in tumor cells, the activated Ras as well as upstream and downstream elements of the Ras-pathway, inhibit (or reverse) PKR activation, thereby allowing reoviral replication resulting in oncolysis (Wilcox *et al.* 2001). The activating mutations in *Ras* have been reported for >30% of tumors. In addition, the mutations in upstream and downstream arms leading to constitutive Ras pathway signaling have been reported for an even greater proportion of tumors (Norman *et al.* 2004). Based on these facts, reovirus has been shown to be effective as an oncolytic agent for a variety of tumors, including malignant glioma (Wilcox *et al.* 2001), breast cancer (Norman *et al.* 2002) and pancreatic cancer (Etoh *et al.* 2003) in animal models.

VSV provides an example of an oncolytic virus where a tumor cell advantage over normal cells has been exploited for selective viral oncolytic activity. All cells exposed to viral infection produce antiviral interferons (IFNs). However, cancer-specific mutations of gene products in the IFN pathway have been reported in tumors (Stojdl *et al.* 2000).

This defect in IFN response against viral infection has been utilized for selective VSV replication and oncolysis of tumors, such as melanoma (Stojdl *et al.* 2000) and colorectal carcinoma metastatic to liver (Shinozaki *et al.* 2005) in mouse models.

In some cases, natural oncolytic activity has been artificially restricted to a particular type of cell, thereby rendering the virus useful for selective treatment of tumors. For example, oncolytic herpes simples virus type 1 (HSV-1) has been exploited for tumor therapy because it can be modified for restricted viral replication in proliferating glioma cells. Of note, one of the advantages of HSV-based oncolytic vectors is the potential use of the antiviral drug acyclovir, should replication become out of control. HSV-1 based vectors have been tested in various phases of clinical trials for glioma with promising results. In addition, oncolytic viral activity of HSV-1 has been combined with the elicitation of an anti-tumor immune response, in order to improve tumor treatment. For example, Wong *et al.* used an oncolytic HSV-1 expressing the pro-inflammatory cytokine IL-12 for treatment of distantly metastatic squamous cell carcinoma (SCC), and observed significantly improved survival in mice with this combination of oncolytic and immune therapy (Wong *et al.* 2004) as compared to oncolytic therapy alone for treating disseminated disease.

A similar strategy based upon a combination of oncolysis and immunomodulation was used with an oncolytic recombinant VSV expressing murine IL-12 (rVSV-IL12). This virus demonstrated a significant reduction in murine squamous cell carcinoma volume as compared to the control virus without IL-12 (Shin *et al.* 2007).

In addition to above listed viruses, conditionally replicative oncolytic adenoviruses (CRAds) have been used for tumor treatment. These vectors have been developed based upon the understanding of aberrant molecular pathways in tumor cells in conjunction with the understanding of Ad biology. For example, the Rb and p53 oncogenes have mutations in many tumors. This fact has been exploited for the generation of an oncolytic Ad vector, delta-24 (Δ24). In this vector, the E1A region that interacts with Rb has been deleted. This virus therefore replicates more efficiently in tumor cells with mutations in Rb as compared to healthy cells (Fueyo *et al.* 2000). Similarly, another Ad genome sequence, E1B 55kDa, which interacts with p53, was deleted to construct a CRAd named *dl*1520 (Onyx-015) (Bischoff *et al.* 1996). This virus replicates in tumors with mutations in p53. However, it is now assumed that in addition to p53, other factors like infectivity and cell permissiveness also contribute to the differential replication of Onyx-015 (Ring 2002). It was determined that the use of Onyx-015 along with chemotherapy might have synergistic effects for tumor treatment (Khuri *et al.* 2000). However, Onyx-015 is not suitable by itself due to limited replication potency. One of the reasons for the limited efficacy of Onyx-015 might be the loss of functions of E1B that are critical for the Ad life cycle, such as mRNA transport and shut-off of host cell protein synthesis (Ring 2002).

Another type of CRAds are those with tissue specific promoters to impose transcriptional limitations for oncolytic replication in specific target cells. For example, cyclooxygenase-2 (Cox-2) has been shown to be highly expressed in a number of epithelial tumors (Lam *et al.* 2007). Based on this consideration, an infectivity enhanced CRAd with the E1 region under transcriptional control of the Cox-2 promoter was constructed. This vector demonstrated potent anti-tumor effects as compared to the wild type vector for pancreatic (Yamamoto *et al.* 2003) and ovarian tumors (Kanerva *et al.* 2004) both *in vitro* and *in vivo*. Another example of transcriptional control of CRAd replication exploits the fact that tumor cell growth is dependent upon neovasularization. For this purpose, vascular endothelial growth factor (VEGF) is produced by tumor cells to drive the angiogenesis. Takayama *et al.* utilized a tropism-modified CRAd in which expression of E1 region, necessary for viral replication, was put under transcriptional control of VEGF promoter. This vector replicated efficiently in lung tumors *in vitro* and *in vivo* (Takayama *et al.* 2007).

Issues pertaining to oncolytic viral therapy

Despite all these developments, many problems have hampered successful utilization of oncolytic viruses for tumor treatment. Upon intra-tumoral or peripheral administration of the oncolytic virus, it was expected that viral progeny would spread to the entire tumor mass and eliminate the tumors efficiently. However, when the first pre-clinical analyses were performed, it was apparent that oncolytic viruses did not spread through the tumor mass as expected. This might be due to the large size of the virus (90 nm for Ad), and physical barriers such as cell-to-cell barriers, basement membranes, necrotic regions and intermixed normal cells (Vile *et al.* 2002).

Another issue that needs to be addressed is the targeting of the virus to specific cells. For example, Ad vectors bind to the coxsackie adenovirus receptor (CAR), which is expressed at high levels in normal tissues of the body such as liver, but at low or negligible level in certain tumors. This results in low viral vector infection efficiency for the tumor cells. In order to achieve the needed infectivity enhancement, viral vectors have been genetically modified. For instance, Krasnykh *et al.* constructed chimeric Ad5/3 vectors, in which the knob domain of Ad5 was replaced by the knob domain of Ad3. This chimeric virus was shown to bind to cells by utilizing receptors other than CAR (Krasnykh *et al.* 1996), resulting in its ability to infect cell lines deficient in CAR-expression. Another example for the Ad vector infectivity enhancement is provided by Wu *et al.*, who constructed Ad vectors with RGD and pK7 motifs in the fiber. It is known that the amino acid sequence arginine-glycine-aspartate (RGD) binds to integrins. Furthermore, it has been demonstrated that poly-lysine sequences (pK7) bind to heparin sulfate-containing receptors. Integrins and heparin sulfate-containing receptors are overexpressed in many tumors. The double modified Ad vector containing RGD and pK7 motifs in the fiber was shown to be capable of infection in both CAR-positive as well as CAR-negative cell lines. The observed infectivity enhancement was a result of the utilization of additional receptors for cell entry by the double modified Ad vectors (Wu *et al.* 2002b).

In addition to the above issues, it has been realized that oncolytic potency of the viral vectors must be determined before these vectors are employed in clinical trials. The oncolytic vectors are usually evaluated in immunodeficient mouse models containing xenografts of human tumors. However, being immunodeficient, these mouse models do not represent the actual scenario in the body of an immunocompetent human patient. In addition, mouse tissues are not very permissive for the replication of human viral vectors such as Ad vectors. In order to overcome these issues, Thomas *et al.* have developed a Syrian hamster model for study of the oncolytic Ad vectors. This model is immunocompetent and permissive to infection by the Ad vectors, thereby mimicking the human physiological system more closely than the mouse models (Thomas *et al.* 2006). However, this model still needs better characterization before its potential can be fully exploited.

In addition to the use of animal models, liver and tumor tissue slices from patients have also been used to evaluate the toxicity characteristics of oncolytic viruses. Since tissue slices can be directly derived from cancer patients, they provide a more physiologically relevant platform for analysis of toxicity of oncolytic viruses (Stoff-Khalili *et al.* 2007b). However, there are practical considerations regarding the availability of fresh tissue slices that are currently limiting their widespread application.

Another method to analyze the characteristics of oncolytic viruses is the use of *in vitro* human cell cultures. However, adherent cell culture is a two-dimensional system as opposed to the three-dimensional tumor environment. Thus,

novel assay systems are being developed to aid in pre-clinical analysis of the oncolytic potency of the viruses. For example, Lam *et al.* have developed a tumor-spheroid three-dimensional system as compared to two-dimensional cell culture mono-layers to measure the viral penetration and oncolytic potency (Lam *et al*. 2007).

Thus, selectively replicative oncolytic viruses are a potent tool for treatment of diseases like cancer. These viruses will be used more widely for treatment once issues related to their oncolytic potency and safety are resolved.

TARGETING OF VIRAL VECTORS

In gene therapy, it is imperative that the therapeutic gene is delivered specifically to the intended target cells. Similarly, the viral vectors that are used for oncolytic therapy must infect and replicate only in the particular cell type that needs to be killed. However, the native tropism of viruses utilized for gene therapy does not necessarily correspond with the desired cell type that needs to be infected. For example, Ads bind to CAR, which is expressed at high levels in normal tissues of the body, such as liver, and not in the intended targets like tumor cells. Therefore, upon Ad vector administration, liver related toxicity can be observed. Similarly, retroviruses are known to infect proliferating cells. Although tumor cells proliferate rapidly, there are other body cells that also undergo proliferation. Thus, retroviral replication must be restricted to tumor cells only and not to normal body cells. Another example is AAV-2, which infects liver cells. This interaction is mediated by heparin sulfate proteoglycan molecules that are present on liver cells. Thus, to use AAV-2 for gene therapy of extrahepatic tissues, its binding to hepatic cells must be perturbed. Therefore, for the development of effective gene therapy viral vectors, the native viral tropism needs to be ablated and viral vectors need to be retargeted to tumor cells.

The targeting of viral vectors can be either transductional or transcriptional. Transductional targeting involves modification of viral tropism whereas transcriptional targeting involves modulation of the viral gene expression such that viral genes are expressed only in desired cell types.

Transductional targeting

Transductional targeting has been achieved through a variety of approaches, including bifunctional adapters and genetic modifications of the viral vector.

Bifunctional adapters for transductional targeting

Bifunctional adapters, as the name indicates, are a combination of two different subunits, one of which binds to the viral vector and the other binds to the target cell. The two different subunits can be attached to each other by either chemical or genetic methods. There are a variety of subunits, some of which will be discussed in more detail below.

Chemically conjugated bifunctional adapters

Due to the technical ease of coupling two subunits by chemical methods, the initial bifunctional adapters contained subunits that were chemically linked. For example, a chemically coupled bispecific antibody conjugate was generated, in which an antibody against Ad was chemically liked to an antibody against epidermal growth factor receptor (anti-EGFR). This bispecific antibody was successfully utilized for targeting Ad vectors to EGFR expressing human glioma cells (Miller *et al.* 1998). However, due to the chemical coupling strategy employed for linking the two subunits, there was variability in the resulting bispecific antibody product, leading to batch to batch variations. Thus, a more consistent production strategy was desired.

Genetically conjugated bifunctional adapters

To circumvent the problems observed with chemical coupling of the subunits, genetic coupling of the subunits constituting the bifunctional adapters was endeavored. For example, an adenobody is a genetic fusion of a single chain antibody (scFv) directed against the Ad fiber knob to a ligand that binds to a target cell. For example, Watkins *et al.* fused a scFv against Ad knob with epidermal growth factor (EGF), which can bind to EGFR on human cells (Watkins *et al.* 1997). Haisma *et al.* further extended the adenobody approach by constructing a bispecific scFv, called a single chain diabody (scDb). For this purpose, a scFv against Ad was genetically fused with a scFV against the EGFR (Haisma *et al.* 2000). Another example of a scDb is for melanoma retargeted Ad vectors, where a scFv against Ad was genetically fused with a scFv against the high molecular weight melanoma antigen (Nettelbeck *et al.* 2004).

In addition to the use of scFc against the Ad knob, other types of subunits with an affinity for Ad knob have been utilized for construction of bifunctional adapters. For example, the ectodomain of the native adenoviral receptor CAR fused to scFvs that target tumor associated antigens has also been exploited for retargeting Ad vectors to specific cells. In this regard, Everts *et al.* fused the ectodomain of CAR, sCAR, with a scFv directed against carcino-embryonic antigen (CEA), which is over-expressed in the adenocarcinomas of the gastrointestinal tract, lung and breast. This bifunctional adapter successfully re-targeted Ad vectors to CEA artificially expressed in the lungs after intravenous administration (Everts *et al.* 2005).

Using these bifunctional adapters, Ad vectors have been efficiently retargeted to desired cells or tissues. In addition, the retargeting and accompanying ablation of native tropism also reduced the Ad vector sequestration in liver, leading to reduced toxicity. However, binding a bifunctional adapter to the viral vector requires an incubation step before infection can be achieved. In addition, even though genetic bifunctional adapter molecules themselves are of a homogenous nature, the incubation of them with Ad vectors will still result in batch-to-batch variations, which are undesirable for clinical application. Moreover, there is always a possibility that the bifunctional adapter does not attach to all the viral sites, thereby sustaining the possibility of viral infection in unintended target cells. In order to resolve these issues, genetic transductional targeting approaches have been developed.

Genetic transductional targeting

A variety of vectors and methods have been used to genetically modify viral vectors in order to achieve the required targeting. For example, Girod *et al.* inserted a 14-amino-acid targeting peptide, L14, into the capsid of AAV-2. The resulting capsid modified virus was demonstrated to efficiently infect previously resistant cell lines that display the integrin receptor recognized by L14 (Girod *et al.* 1999). Although insertion of a targeting moiety against a particular target cell receptor into the viral capsid is an efficient way of targeting the virus, it is very time consuming to incorporate a specific targeting ligand into the viral capsid for a cell type of interest. Thus, a more general targeting approach might be more beneficial, especially for screening purposes. In this regard, Ried *et al.* incorporated the immunoglobulin G (IgG) binding domain of protein A, Z34C, into the AAV-2 capsid. The resulting AAV-2 mutants could be targeted to distinct hematopoietic cell lines using an antibody against CD29 (β_1-integrin), CD117 (c-kit receptor) and CXCR4 (Ried *et al.* 2002). Another example of a general targeting approach is provided by genetically modified Ad vectors. In this regard, Noureddinni *et al.* also fused the Fc-binding domain of *Staphylococcus aureus* protein A into a chimeric fiber expressed on Ad vectors. This modified Ad vector can now be utilized to infect a broad range of target cells, depending on the monoclonal antibody that is coupled to the Fc-binding domain on the Ad vector

(Noureddini *et al.* 2006).

In addition to genetically incorporating the targeting ligands in the capsid of the viral vectors, another approach that has been proposed is pseudotyping. It involves substituting the receptor binding proteins of one virus for those of another virus. For example, an AAV-2 genome encapsidated into a parvovirus B19 capsid can provide a new tool for AAV-2 targeting to specific cells, based on the natural tropism for human erythroid progenitor cells of parvovirus B19 (Ponnazhagan *et al.* 1998).

One of the most advanced forms of genetic transductional targeting is to directly incorporate antibodies recognizing the target cell antigens into the viral capsid. This has recently been achieved for Ad vectors. Hedley *et al.* genetically incorporated a scFv into the fiber of Ad vectors and demonstrated successful targeting to receptors on the surface of target cells (Hedley *et al.* 2006). It will be of interest to see the targeting capacity of these genetically modified vectors in an *in vivo* context, and determine their translational potential.

Similar genetic approaches have also been applied for targeting of other viruses. For example, scFv against CD38 and EGFR have been genetically incorporated into measles virus (MV) (Nakamura *et al.* 2005). More recently, Hasegawa *et al.* genetically modified the tropism of MV for targeted virotherapy of ovarian cancer. For this purpose, they incorporated the scFv specific for α-folate receptor (FRα), which is over-expressed on 90% of nonmucinous ovarian cancer, into the attachment protein of MV. This virus reduced the tumor volume and also increased the overall survival of mice as much as the parental virus, but without the side effects of the untargeted virus (Hasegawa *et al.* 2006).

Transcriptional targeting in combination with transductional targeting

The above examples illustrate the approaches that have been developed for targeting viral vectors to specific cells. However, a strategy to supplement the tranductional targeting is to involve transcriptional targeting as well. For this purpose, cell specific promoter elements have been incorporated into the genome of viral vectors to limit viral gene expression in specific cell types. For example, Muller *et al.* used AAV-2 devoid of binding to their primary receptor heparin sulfate proteoglycan. In this virus, they incorporated a luciferase reporter gene under the control of 1.5-kb cardiac myosin light chain promoter, fused to the cytomegalovirus immediate early enhancer. The combined transductional and transcriptional targeting with this virus resulted in efficient gene transfer to cardiac cells *in vivo* and also had a significantly reduced hepatic sequestration (Muller *et al.* 2006).

Another example for combined transductional and transcriptional targeting is provided by Ad vector targeting to endothelial cells. To achieve this targeting, Reynolds *et al.* utilized a chemically linked bifunctional adapter. For this, a Fab fragment against Ad knob was chemically coupled to an antibody against angiotensin converting enzyme (9B9), which is a membrane-bound ectopeptidase expressed on pulmonary vascular endothelium. For transcriptional targeting, the promoter for vascular endothelial growth factor receptor type-1 (flt-1), which has high activity in endothelial cells, was utilized to drive the expression of a luciferase reporter gene. The combined transductional and transcriptional approaches resulted in a synergistic 300,000-fold improvement in the selectivity of transgene expression for lungs as compared to the liver, which is the usual vector sequestration site (Reynolds *et al.* 2001). Thus, combined targeting approaches have been shown to be useful for cell type specific viral vector delivery and therapeutic gene expression, for improved gene therapy.

Targeting of the viral vectors to the appropriate cells is crucial for development of an efficient gene therapy regimen and as illustrated by above examples, many unique strategies have been developed for this purpose. Though specific target cell delivery increases the therapeutic gene transfer to target cells, unfortunately an immune response elicited against the viral vector still limits full utilization of targeting approaches.

STRATEGIES FOR IMMUNE SYSTEM EVASION BY VIRAL VECTORS

Viral vectors utilized for gene therapy are recognized as foreign by the host in which they are injected, and are therefore countered by an immune response. The immune response consists of innate and adaptive responses. The innate response is elicited upon recognition of the foreign viral capsid components by the immune system. The innate response leads to clearance of the viral vector before the viruses have had a chance for primary infection (Bessis *et al.* 2004; Muruve 2004). This diminishes the efficiency of the transgene delivery to target host cells. Following successful viral infection of host cells, the adaptive arm of the host immune system is activated against the viral proteins that are produced in the host cells and the therapeutic gene if it is foreign to the host. The adaptive response also results in the development of immune memory, which further limits viral re-administration (Bessis *et al.* 2004). Also, pre-existing immunity against the viral vector further compounds the problem of efficient therapeutic transgene delivery by the viral vector. For example, Ads are one of the causative agents of the "common cold" and thus, many patients have pre-existing humoral immunity against the viral vector. This leads to rapid clearance of the therapeutic viral vector from the blood stream, prevents re-administration of the viral vector and results in overall reduction in the efficacy of the viral vector based gene therapy. This suggests that suppression or avoidance of the immune system would be needed to achieve sufficient viral vector based therapeutic effects. However, the immune response generated against the viral vector and/or the delivered transgene can also be exploited for manipulating the host immune system in developing an effective immune response against tumor cells. The following examples illustrate these points in more detail.

Immuno-suppression

To circumvent the immune system mediated removal of the viral vector, a variety of approaches have been developed. In this regard, immuno-suppressants have been used to blunt the immune system of the host, thereby increasing the transgene delivery and expression by the viral vector. For example, Jooss *et al.* administered an Ad vector along with different doses of cyclophosphamide, which suppresses T cells. They demonstrated an effective blockade of both T and B cell responses in the liver and the lungs of C7BL/6 mice using this strategy. This resulted in prolonged transgene expression, reduced inflammation and allowed re-administration of the Ad vector (Jooss *et al.* 1996). However, the use of immunosuppressive drugs, which diminish the immune response capacity of the patient against foreign pathogens, causes concern.

Another strategy that has been utilized for immune system modulation involves perturbation of the host immune system at the level of cross-talk among different immune cell types. Disruption of the co-stimulatory interactions between antigen presenting cells (APCs) and B and T cells has been shown to be successful for reducing the cellular as well as humoral response generated against the viral vector. APCs present processed foreign antigens in association with major histocompatibilty complex (MHC) molecules to T cells for their activation. In addition to the antigenic peptide and MHC interaction with the T cell receptor (TCR), other co-stimulatory molecules also play an important role in T cell activation. In this regard, B7 proteins on APCs bind to CD28 on T cells, providing a critical second co-stimulatory signal, especially for the primary response

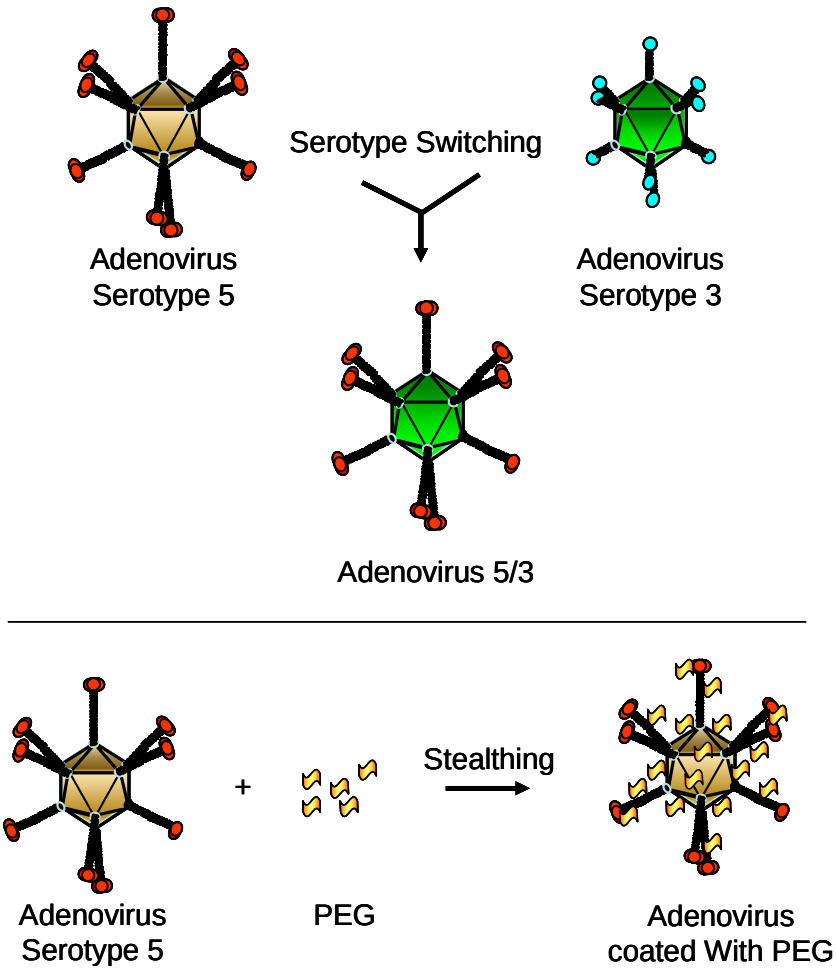

Fig. 3 Strategies employed for immune system evasion. Pre-existing immunity against gene therapy vectors is a major limitation to effective gene transfer. Strategies to overcome this hurdle include serotype switching (top) and physical masking of antigenic epitopes (bottom). Top: Serotype switching encompasses the construction of vectors containing capsid proteins from different serotypes. For example, a chimeric Ad5 vector expressing hexon protein of Ad3 was constructed. This vector was not recognized by antibodies against the hexon protein of Ad5, thereby allowing vector re-administration. Bottom: Alternative strategies have included physical masking of antigenic epitopes on viral vectors. For example, poly(ethylene glycol) molecules can be chemically conjugated to Ad vectors, which protect the vectors against antibody recognition.

of the naïve T cells to novel antigens. B7 also binds to CTLA4 on the T cell surface, which primarily dampens T cell activation. Thus, blocking the interaction of B7 with CD28 will inhibit T cell priming, which will inhibit downstream immune responses activated by T cells. In this regard, it has been shown that the extracellular domain of CTLA4 fused to an immunoglobulin IgGFc domain (CTLA4Ig) binds to B7 with 20-fold higher affinity as compared to CD28. A consequence of the interaction of antigen-MHC with TCR in the absence of B7-CD28 interaction can be the induction of T cell energy or prolonged unresponsiveness (Kay *et al.* 1997).

Another immune system interaction that has been disrupted is the interaction between activated T cells and B cells. Activated T cells express CD40, which binds to CD40 ligand on the surface of B cells, which is critical for the development of a humoral B cell response. This interaction can be blocked by a monoclonal antibody, MR-1, against CD40 ligand. Blockade of this interaction results in immunodeficiency in antibody response (Kay *et al.* 1997). A combination of CTLA4Ig with MR-1 has been utilized for suppressing the host immune system. For example, it has been shown that administration of MR-1 protein along with CTLA4Ig allowed for re-administration of AAV in lung (Halbert *et al.* 1998) and Ad in the liver (Kay *et al.* 1997).

An alternate strategy that has been utilized for immunosuppression is incorporation of immune system suppressor genes in the viral vector itself. Immune system suppressing genes have been used to blunt the immune response even when the viral vector encoded proteins are produced in the host cells. For instance, Haralambieva *et al.* incorporated the P gene from a wild type measles virus (MV) strain into an oncolytic MV. The P gene product inhibits interferon (IFN) induction and/or response. The resulting chimeric oncolytic virus armed with the P gene exhibited reduced IFN sensitivity, diminished IFN induction capacity and enhanced oncolytic potency as compared to the control oncolytic MV (Haralambieva *et al.* 2007).

Modification of the viral vector for immune system evasion

In order to prevent immune rejection of the viral vectors, various strategies have been employed for their modification in addition to immunosuppression. One of the strategies involves deletion of the unnecessary viral genome sequences resulting in reduced viral protein expression. The reduced viral protein production results in less immune stimulation. This strategy has been successfully applied for reducing the immune response against the viral vector. For example, as described in another section, gutless Ad vectors devoid of most of the genome sequences have been reported to have improved transgene expression and enhanced safety profile (Morsy *et al.* 1998; Schiedner *et al.* 1998).

Another strategy for immune evasion is based upon serotype change of the viral vectors. Serotype specificity is one of the ways to classify subtypes of viruses. Per definition, antibodies generated against one viral serotype do not recognize another viral serotype. Based on this consideration, Riviere *et al.* demonstrated that different recombinant AAV serotypes, AAV type 1, 2 and 5, can be utilized for repeated cross-administration for transgene delivery (Riviere *et al.* 2006). This is because pre-existing immunity against one serotype of a viral vector does not prevent administration of another serotype of that viral vector. Another such example is provided by Ad vectors that express capsid proteins derived from two different serotypes, so called chimeric vectors. In this regard, it has been reported that the major antibody response is generated against the hexon capsid protein of Ad vectors. Based on this consideration, Wu *et al.* constructed a chimeric adeno-virus, Ad5/H3, by replacing the Ad5 hexon gene with the hexon

gene of Ad serotype 3 (**Fig. 3**). They demonstrated that antibodies against either the parent virus with the Ad5 hexon protein (Ad5/H5) or the chimeric virus with Ad3 hexon protein (Ad5/H3) did not cross-neutralize the other virus. In addition, pre-immunization of C57BL/6 mice with either of the viruses did not prevent subsequent infection by the other virus (Wu *et al.* 2002a). Thus, serotype switching strategies can be utilized for re-administration of the viral vectors. However, for each re-administration, a vector with different serotype will be required. Generation of these serotype viral vectors requires much effort and they may not transduce the same target cell population.

In addition to the above genetic modification strategies, viral vectors have also been modified through chemical strategies, most notably by the use of poly(ethylene glycol) (PEG) to mask the antigenic epitopes on the viral surface. This is also known as 'stealthing' (**Fig. 3**). PEG is a hydrophilic molecule, which physically masks the capsid proteins, thereby resulting in reduced innate immune response generated against the viral vector (Mok *et al.* 2005). Croyle *et al.* showed that PEGylated gutless Ad vectors could be re-administered with efficient transgene expression. Thus PEGylation can be utilized for improving the safety and efficacy profile of the viral vectors (Croyle *et al.* 2005). However, an immune response will still be generated against the new viral progeny produced in infected cells.

Recently, PEGylation-based immune evasion has been combined with molecules utilized for retargeting of the viral vectors to the desired cell types. For example, folate was chemically conjugated to PEG. The resulting folate-PEG was subsequently coupled to Ad vectors. This approach increased the transgene expression in folate receptor over-expressing cell line (KB cells) as compared to the folate receptor deficient cell line (A549 cells). In addition, PEGylation significantly reduced the innate immune response against the Ad vector (Oh *et al.* 2006). Thus, this combinatorial approach efficiently protects viral vectors from the innate immune system and also aids in efficient transgene delivery to specific target cells.

The examples listed above illustrate the various strategies that have been utilized for protecting the viral vector from the host immune system. However, the immune response generated against the viral vector and/or the delivered transgene can also be utilized in substituting immunity against the tumor cells. Although in general an immune response should be avoided to achieve a sufficient therapeutic effect, in the context of cancer immunotherapy this response is actually desired to efficiently utilize the capacity of the host immune system to kill the tumor cells. In this regard, viral vectors have been utilized for developing immunity against tumor-associated self antigens and thereby break tolerance. For example, AAV-2 was utilized to deliver BA46 to dendritic cells. BA46 is a membrane-associated glycoprotein that is expressed in most breast tumor cells, but not in general hematopoietic cell populations. The AAV-2 mediated BA46 delivery to dendritic cells resulted in generation of cytotoxic T lymphocytes against BA46 populations, which could potentially kill the breast cancer cells (Liu *et al.* 2005). Another example is provided by an Ad vector encoding HER2. The HER2/*neu* oncogene encodes for a protein p185 (C-erbB2). This protein is over-expressed in 30-50% of human breast cancer and in several other types of carcinomas. p185 has high oncogenic potential and its increased expression correlates with tumor aggressiveness. Ad-HER2 was injected intra-muscularly in BALB/c mice that are transgenic for the transforming form of the *neu* oncogene. These mice spontaneously develop carcinomas in all mammary glands. The Ad-HER2 vaccination resulted in both T and B cell responses against HER2, thereby preventing tumorigenesis (Gallo *et al.* 2005). Thus, viral vectors can potentially be utilized for generating immune response against the tumor cells.

The above examples highlight a few of the strategies that have been successfully used to counter the immune response that is generated upon viral vector administration such as immunosuppression, expression of immune suppression genes and genetic as well as chemical vector modifications. In addition, the immune response generated against the viral vector and its transgene has been exploited for developing patient's immunity against the tumor cells.

CELL-BASED STRATEGIES FOR CANCER GENE THERAPY

In addition to the virus-based strategies described above, viruses have also been utilized for cell-based strategies aimed at cancer gene therapy. Many of these strategies are centered on using cells as factories to produce angiogenesis inhibitors or cytokines that prime the immune system. Other strategies are aimed at using cells as "trojan horses" to deliver suicide genes or oncolytic viruses directly within the tumor stroma. Cell vehicles used as factories can result in the localized and sustained production of therapeutic proteins, the length of which depends on the type of vectors used for gene transfer, the cellular targets transduced, and the immunogenicity of the therapeutic proteins produced.

Therapeutic effector molecules for cell-based therapy

Angiogenesis inhibitors, such as angiostatin (O'Reilly *et al.* 1994) and endostatin (O'Reilly *et al.* 1997), are effective at limiting tumor growth and metastasis, but the fact that micrometastatic lesions can lay dormant may require continuous production to prevent future tumor outgrowth (Scappaticci 2002). Gene therapy approaches may be ideal for these situations, since these strategies allow for localized and sustained production, and avoids the need for the doses required for systemic efficacy (Persano *et al.* 2007). Mesenchymal stem cell mediated delivery of IL-12 was recently reported to reduce the formation of lung metastasis in a murine melanoma model, although NK and T cell mediated responses were also involved in the outcome (Elzaouk *et al.* 2006). A recent study by Jin *et al.* describes the combined use of an Ad vector that targets expression of an anti-angiogenic factor to the tumor endothelium along with a conditionally-replicating oncolytic Ad vector containing a tumor-specific promoter (Jin *et al.* 2005). A similar approach can be envisioned, using cell-mediated delivery of both therapeutic and oncolytic vectors. Combined therapeutic strategies for a disease marked by such vast epigenetic differences will likely be required. The true potential of angiogenesis inhibitors may be in the fact that they allow time for additional therapeutic avenues to take effect.

Cytokines are also favored as key therapeutic products for cell vehicle mediated delivery. As with angiogenesis inhibitors, large doses are often required to achieve therapeutically relevant concentrations. However, unlike angiogenesis inhibitors, elevated cytokine concentrations can have adverse effects (Lejeune *et al.* 1998; Neri *et al.* 2006). Thus, cellular vehicles may also serve to express and secrete the requisite cytokines for localized production at concentrations that limit untoward complications to the host. These cellular factories also abrogate the need for recombinant protein production and purification techniques. Minuzzo *et al.* recently provided a detailed review of the combined use of viral vectors with cell-mediated delivery of cytokines (Minuzzo *et al.* 2007).

Cancer gene therapy studies have also evaluated the use of prodrug activating enzymes, or suicide genes, that convert an exogenously provided substrate into a cytotoxic molecule. The herpes simplex virus thymidine kinase gene (HSV-TK) acts as a suicide gene in the presence of the guanosine analog, gancyclovir (GCV) (Elion 1980; Moolten 1986). Cell vehicles that express these suicide genes and engraft tumors can cause a 'bystander effect', or collateral damage to surrounding tumor cells upon addition of the prodrug (Freeman *et al.* 1993). Tumor cells, endothelial cells, progenitor cells, and mesothelial cells have all been evaluated as vehicles to deliver the HSV-TK/GCV medi-

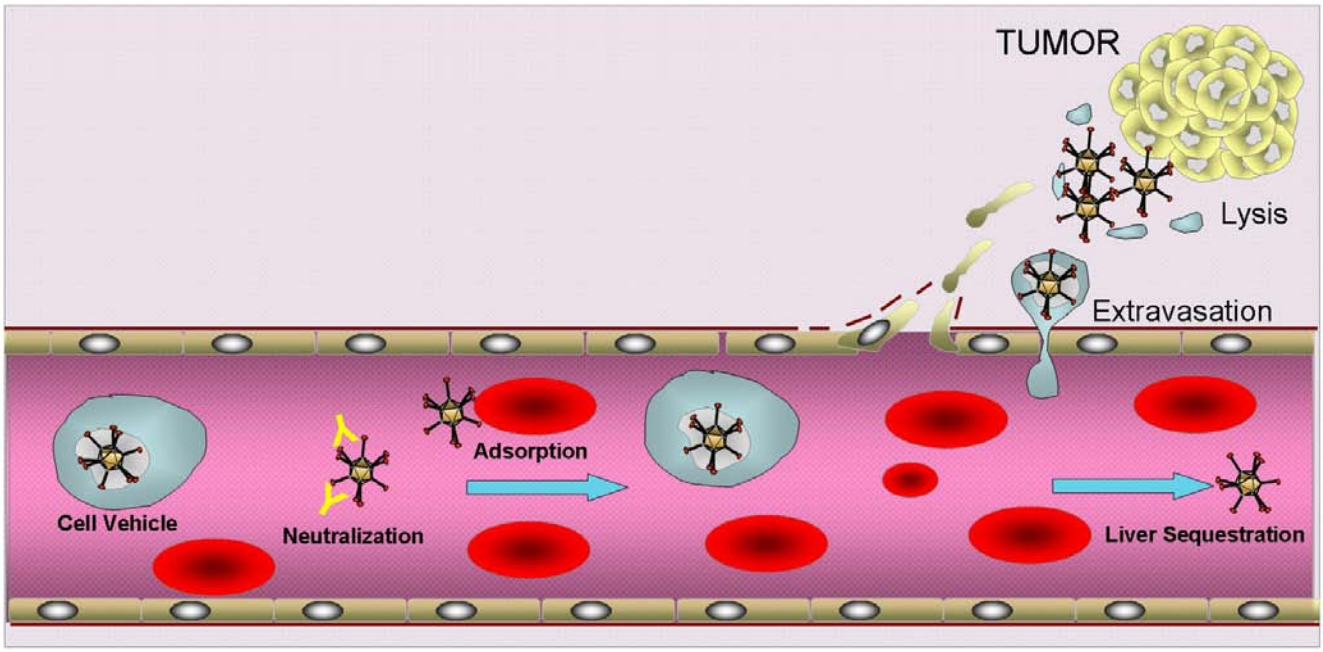

Fig. 4 Fate of systemically delivered Ad vectors. Systemically administered Ad vectors are not able to escape the circulatory system and are thus rapidly sequestered by cells of the reticuloendothelial system. Furthermore, Ad targeting is limited by soluble immune factors, such as complement and neutralizing antibodies, and non-specific interactions with erythrocytes, neutrophils, and monocytes. In contrast, cells that have intrinsic or engineered targeting activity can be loaded with Ad vectors and serve as site-specific delivery vehicles that protect virions from inactivation, while amplifying the payload in transit.

ated bystander effect to tumors (Rancourt *et al.* 1998; Coukos *et al.* 1999; Pereboeva *et al.* 2003; Rancourt *et al.* 2003).

Recent studies have centered on the use of cell vehicles to deliver oncolytic adenovirus vectors. This strategy avoids complications and the marked inefficiency associated with systemic introduction of viruses, such as preexisting neutralizing antibodies, non-specific vector sequestration in the liver or blood, and the inability to cross the endothelial barrier (**Fig. 4**) (Chirmule *et al.* 1999; Tsujinoue *et al.* 2001; Shayakhmetov *et al.* 2004; Franceschi 2005; Shayakhmetov *et al.* 2005). As described above, the list of naturally occurring, or recombinant oncolytic viruses includes adenovirus, herpes (Martuza *et al.* 1991), vaccinia, reovirus (Coffey *et al.* 1998), poliovirus, and Newcastle Disease Virus (Cassel *et al.* 1965; Martuza *et al.* 1991; Bischoff *et al.* 1996; Coffey *et al.* 1998; Timiryasova *et al.* 1999; Gromeier *et al.* 2000). Various cellular vehicles have also been employed to deliver these agents to tumors. Tumor cells infected with oncolytic parvovirus (Raykov *et al.* 2004) or Ad (Garcia-Castro *et al.* 2005) vectors have been shown to engraft and deliver the oncolytic payload to preexisting metastatic nodules. Others have used mesenchymal progenitors cells to deliver oncolytic agents to lung (Stoff-Khalili *et al.* 2007a) or intraperitoneal (Komarova *et al.* 2006) tumor xenografts. Cytokine induced killer cells have inherent tumor killing activity that is enhanced if the cells are preloaded with oncolytic vaccinia virus (Thorne *et al.* 2006). Iankov *et al.* recently reported the comparison of several cell vehicles as oncolytic measles virus carriers (Iankov *et al.* 2007). This strategy transferred the virus via a heterofusion mechanism, even in the presence of neutralizing antibodies, further demonstrating the true potential of this approach.

Cell types used in cell-based therapy

Along with the genetic payload to be used, the cell types suited or available for use as vehicles for cancer gene therapy will be critical. Different cell types have unique characteristics that may be required for efficient cancer gene therapy. In general, ideal cell vehicles are non-invasively accessible, can be purified and expanded to therapeutic levels, are susceptible to genetic manipulation, and home and engraft therapeutically-relevant target sites. Cell size is often a limiting factor due to the fact that systemic administration requires that the cells are capable of circulating through the lung microvasculature. Thus, the cells meeting most of the cell vehicle criteria are of hematopoietic origin, as these cell types are innately geared for systemic circulation. Further, many of the other characteristics defining ideal cell vehicles are natural properties of hematopoietic cells, including their ability to infiltrate tumor tissues.

Of the many leukocyte subsets found within the tumor stroma, tumor-associated macrophages (TAMs) are the most abundant, and are typically associated with poor prognosis (O'Sullivan *et al.* 1994; Leek *et al.* 1996; Takanami *et al.* 1999). Macrophages are essential components of innate immunity, acting as both antigen presenting and effector cells that protect the body against invading pathogens. Macrophages arise from progenitors in the bone marrow, entering circulation as promonocytes, where they differentiate into monocytes. Monocytes infiltrate tissues, further differentiating into resident macrophages. Macrophage infiltration and accumulation is a normal part of the inflammatory processes resulting from wounds and infection, as well as chronic inflammatory disease. Tumor cells secrete chemotactic molecules such as CCL2, macrophage-colony stimulating factor, and vascular endothelial growth factor that act to recruit TAM precursors. The tumor cells also secrete cytokines that polarize TAM into type II macrophages, which act to suppress adaptive immunity (reviewed by Mantovani *et al.* (2002)). Hypoxic conditions within tumors also induce expression of TAM genes associated with tumor cell proliferation, invasiveness, and angiogenesis (Murdoch *et al.* 2005). Although TAM are localized at the site of the tumor and play a part in tumor development, they lack the ability to home to tumors if isolated and systemically re-infused (Wiltrout *et al.* 1983; Ben-Efraim *et al.* 1994).

Many other leukocyte subsets are also found within the tumor stroma, including tumor-infiltrating lymphocytes (TILs). TILs have been shown to have either tumor-suppressing or tumor-promoting activity. $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) suppress antitumoral immunity and thus promote tumor growth, while $CD8^+$ cytotoxic T

lymphocytes (CTLs) have direct tumor cell killing activity (Chen *et al.* 2005; Nishikawa *et al.* 2005). Unlike TAMs, TILs can be isolated, expanded *ex vivo*, and home to tumors when systemically reimplanted into the patient. This adoptive transfer approach has recently been shown to be an effective strategy for the treatment of melanoma. Interestingly, unmodified (Dudley *et al.* 2002) tumor-reactive T cells, and T cells engineered with viral vectors to be tumor reactive (Morgan *et al.* 2006) have both demonstrated effective tumor regression in melanoma patients.

Several other non-hematopoietic cell types have also been evaluated as cell vehicles for cancer therapy. Progenitor cells are widely used for this strategy. These cells are rapidly recruited to sites of injury where they differentiate into the cellular components required to repair the damaged tissue (Mackenzie *et al.* 2001). The architecture of a rapidly developing tumor closely resembles damaged tissue in that it is often disorganized, inflamed, and hypoxic (Haroon *et al.* 2000). Not surprisingly, mesenchymal and endothelial progenitor cells are recruited to the site of the tumor and can contribute to malignant growth (Studeny *et al.* 2004).

The specific cell types used will largely depend on the types of tumors being targeted and the types of therapeutics intended for delivery. Systemic injection of cells, unless specifically targeted to the lung, should be restricted to hematopoietic cell lineages that can circulate through the microvasculature. Locoregional, or intratumoral injection of cell vehicles may utilize additional cell types. In the rare circumstances in which natural tumor-homing T cells are attainable, delivery of lytic viruses may not be the best option, as these cells have inherent tumor-killing activity. As previously mentioned, many non-tumor cells contribute to tumor cell growth. Cell mediated delivery of agents that target elimination of Tregs or TAMs within the tumor may also prove to be therapeutically useful.

FUTURE PERSPECTIVES

The above mentioned examples highlight the crucial role viral vectors play in gene therapy applications. However, problems related to efficient delivery of the transgene to target cells, long-term transgene expression and immune responses against the viral vector and infected cells have prevented utilization of the full potential of viral vectors. As noted above, various strategies have been employed to enhance the transgene delivery and expression and reduce viral toxicity. In future, continued progress in these respects will further improve overall efficiency of the viral vector based gene therapy.

Cell based therapy has utilized the many advances in viral vector mediated gene expression technology for concentrated, but localized delivery of therapeutic products. Although the idea of cell-based delivery of therapeutics has been around for quite a while, practical application has been limiting. Realization that particular cell types have true homing potential has led to revitalized interest in this technology. Much of the transcriptional and targeting knowledge obtained for both viruses and cells can now be combined for multifaceted cancer treatment approaches.

One of the interesting aspects related to tumor therapy is that combination of gene therapy with radiotherapy (Rogulski *et al.* 2000) or chemotherapy (Khuri et al. 2000) has shown synergistic effects for tumor treatment. Thus, a combinatorial approach has been determined to be optimal for tumor treatment. Therefore, most likely in future viral vectors will be combined with both existing treatments for cancer, as well as new treatment opportunities offered by for example, nanotechnology. As an example, gold nanoparticles (AuNPs), can be used for hyperthermic tumor cell ablation using laser irradiation (O'Neal *et al.* 2004). Everts *et al.* have attached AuNPs to Ad vectors to deliver these nanoparticles specifically to tumor cells (Everts *et al.* 2006). This complex of Ad vectors with AuNPs can potentially be used for simultaneous tumor treatment with gene therapy and nanotechnology approaches. These viral vectors with coupled nanoparticles have been previously defined as viro-nano therapy agents (Saini *et al.* 2006).

In conclusion, viral vectors as well as genetically modified cells are important for cancer gene therapy. Technological advances will further increase the utility of viral vectors for efficient gene and cell therapy in future, and much progress can be expected in the coming years, now that major roadblocks have been identified and strategies to overcome these roadblocks have shown promise in pre-clinical models.

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Targeting Nanoparticles to Tumors using Adenoviral Vectors

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ABSTRACT

Development of novel therapies remains essential for treatment of cancer; in this regard, nanotechnology holds great promise. For example, tumor imaging opportunities have expanded by the development of quantum dots (QDs), and novel tumor treatment opportunities are exemplified by the use of gold nanoparticles (AuNPs). However, for all these applications of metal nanoparticles, selective tumor targeting is crucial for successful clinical application. Considering the progress made in targeting adenoviral (Ad) gene therapy vectors to tumors, we herein aim to couple metal nanoparticles to targeted Ad vectors to achieve selective tumor accumulation. We demonstrate that metal nanoparticles such as QDs and AuNPs can indeed be coupled to Ad vectors, without compromising viral infectivity, retargeting ability or function of the nanoparticles. This innovative combination strategy is therefore expected to lead to the development of a unique methodology for cancer detection and treatment.

Keywords: adenovirus, quantum dots, gold nanoparticles, targeting, imaging

1 INTRODUCTION

Despite advances in detection and treatment of cancer, development of novel therapies remains essential in the continuing battle against this disease. In this regard, nanotechnology holds great promise for the detection and treatment of cancer. For example, tumor imaging opportunities have expanded by the development of quantum dots (QDs) for fluorescence based detection [1], or magnetic nanoparticles for magnetic resonance imaging applications [2]. Novel tumor treatment opportunities are exemplified by the use of gold nanoparticles, which upon laser irradiation will heat up and kill neoplastic cells via hyperthermia [3,4]. However, for all these applications of metal nanoparticles, selective tumor localization is crucial for successful clinical application.

In this respect, great progress has been made in targeting gene therapy vectors to tumors. In particular, a virus that causes the common cold – adenovirus (Ad) – has been used in targeted gene therapy for cancer [5]. For example, our laboratory has developed bi-functional adapter molecules, which bind with one domain to the virus and to tumor-associated antigens (TAAs) with the other. We have previously established that these adapter molecules are able to mediate Ad vector targeting to TAAs *in vitro* and to TAAs expressed in the pulmonary vasculature after systemic administration *in vivo* [6]. Importantly, it has also recently been demonstrated that the utility of adapter molecules extends to Ad vectors targeted to TAA-expressing tumors and hepatic metastases, even when delivered systemically (Dr. H.R. Herschman, UCLA, personal communication, manuscript submitted). We therefore aim to couple metal nanoparticles to Ad vectors that are targeted to tumor cells using bi-functional adapter molecules, in order to achieve their selective tumor accumulation. This combination of novel nanotechnology developments with gene therapy targeting strategies is expected to lead to the development of a multi-pronged approach for cancer detection and treatment.

2 EXPERIMENTAL SECTION

2.1 Cell Culture

HEK-293 cells were obtained from Microbix (Toronto, Canada), MDA-MB-361 cells were obtained from ATCC (Manassas, VA, USA) and MC38 cells stably transfected with carcinoembryonic antigen (CEA), MC38-CEA-2, were kindly provided by Dr. Jeffrey Schlom, National Cancer Institute (Bethesda, MD). All cells were maintained in DMEM:Ham's F12 (1:1 v/v, Mediatech, Herndon, VA) medium, containing 10% fetal bovine serum (Hyclone, Logan, UT), 2 nM L-glutamine, 100 IU/mL penicillin and 25 μg/mL streptomycin (all Mediatech). Medium for MC38-CEA-2 cells additionally contained 500 ug/mL G418 (Mediatech). Cells were grown in a humified atmosphere with 5% CO2 at 37 °C.

2.2 Construction, Production and Purification of Bi-Functional Adapter Molecules

Bi-functional fusion proteins capable of retargeting Ad vectors to either the tumor-associated antigen carcinoembryonic antigen (CEA) or c-erbB2 (HER2/neu) were constructed, consisting of the ectodomain of CAR including its own leader sequence (aa 1-236), followed by a 5-aa peptide linker (GGPGS), a 6-histidine tag (for detection/purification), followed by either the anti-CEA single chain antibody MFE-23 (a kind gift from Dr. Kerry Chester, London, UK) or the anti-c-erbB2 antibody C6.5 (provided by Dr. J.D. Marks, Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, CA). To construct sCAR-MFE and sCAR-C6.5, first, cDNA encoding sCAR followed by the 6-his tag was amplified from pFBsCAR6hTf [7], introducing a HindIII (5') while maintaining the BamHI (3') restriction site. Second, the scFvs MFE-23 and C6.5 were amplified by PCR introducing a BamHI (5') and XhoI (3') restriction site. Both sCAR and scFv PCR products were simultaneously ligated into the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA), digested with HindIII and XhoI restriction enzymes, thereby constructing pcDNA/sCAR/6h/MFE and pcDNA/sCAR/6h/C6.5. The constructed plasmids were verified by sequencing. HEK-293 cells were stably transfected with PvuI linearized plasmid using Superfect transfection reagent (Qiagen,Valencia, CA, USA), and clones were selected for high production and secretion of protein in the supernatant. After expansion of a positive clone, media was collected and protein was purified by immobilized metal-affinity chromatography (Ni-NTA Superflow, Qiagen), followed by dialysis against PBS.

2.3 Adenoviral Vectors

For labeling Ad vectors with quantum dots we utilized a virus with a biotin acceptor peptide genetically incorporated into the hexon capsid protein, generously provided by Dr. Michael A. Barry, Baylor College of Medicine [8]. This virus is metabolically biotinylated upon replication, allowing the coupling of streptavidin-labeled molecules, particles or complexes. For labeling Ad vectors with gold nanoparticles we utilized a virus with a six-histidine motif genetically incorporated into the hexon capsid protein, generously provided by Dr. Hongju Wu, University of Alabama at Birmingham [9], allowing coupling of Ni-NTA-labeled molecules, particles or complexes. To produce the viruses, HEK-293 cells were infected using medium containing 2% fetal bovine serum; following overnight incubation regular 10% medium was added to the cells and incubated until a total cytopathic effect was observed. Cells were harvested, frozen and thawed four times, and virus was purified using standard CsCl purification methods. Viral particle number was determined by measuring absorbance at 260nm using a conversion factor of 1.1×10^{12} viral particles per absorbance unit [10].

2.4 Labeling Ad Vectors with Quantum Dots

QDs labeled with streptavidin on their surface (655 nm, Invitrogen, Carlsbad, CA) were incubated with Ad vectors expressing biotin molecules on their surface in a QD:Ad ratio of 1250 (mole:particle), before being added to the c-erbB2-expressing MDA-MB-361 breast cancer cells. Cells were plated the prior day in 2-well Lab-Tek™ Chamber Slides™ (Nalge Nunc International, Rochester, NY) at a concentration of 25,000 cells per well. The Ad-QD complex (MOI 5,000 particles/cell) was targeted to c-erbB2 by adding a final concentration of 1 ug/mL of the previously described bi-functional adapter molecule sCAR-C6.5 to the reaction mixture [11]. The Ad-QD-sCAR-C6.5 complexes, or QDs by themselves, were incubated with cells for 30 min at 4 °C, after which unbound complexes were removed via washing. Cells were subsequently incubated at 37 °C for 30 minutes. Cells were then washed, fixed in neutral-buffered formalin, washed again, embedded in 90% glycerol and imaged utilizing Dual Mode Fluorescence (CytoViva Inc, Auburn, AL).

2.5 Labeling Ad Vectors with Gold Nanoparticles

Ni-NTA-labeled gold nanoparticles (AuNP; Nanoprobes, Yaphank, NY) were incubated with Ad vectors (1×10^{12} viral particles total) presenting a six-histidine motif on their surface and carrying luciferase as a transgene in a AuNP:Ad ratio of 2,000 (particle:particle). As a control, AuNP were incubated with Ad vectors lacking a six-histidine motif and Ad vectors were incubated without AuNP present. AuNP-labeled Ad vectors were separated from unreacted reagents in a CsCl density gradient. Viral particle number was again determined as described above.

To assess Ad retargeting, CEA-expressing MC38-CEA-2 colon cancer cells cells were plated in triplicate at a density of 1×10^5 cells/well in 24-well plates. The following day, 1×10^7 viral particles (MOI 100 particles/cell) were incubated for 15 min at room temperature with 75 ng of the previously described sCAR-MFE [6], before being added to the cells in medium containing 2% fetal bovine serum. After 2 hours of incubation, medium containing the virus was removed and replaced with regular growth medium. Cells were incubated for an additional 22 hours and were subsequently washed with PBS and lysed using Reporter Lysis Buffer (Promega, Madison, WI). After one freeze-thaw cycle, luciferase activity was measured using the Luciferase Assay System (Promega), according to manufacturer's instructions.

3 RESULTS & DISCUSSION

We herein aimed to demonstrate that targeted Ad vectors can serve as a platform for tumor-selective delivery of metal nanoparticles, providing either imaging or therapeutic properties, or both. This would allow a potential combination of nanotechnology and gene therapy approaches for the imaging and treatment of cancer. We therefore analyzed whether delivery of nanoparticles inside tumor cells was feasible, and whether infection of tumor cells with nanoparticle-labeled Ad vectors would still result in transgene expression.

First, we coupled streptavidin-labeled QDs to biotinylated Ad vectors, and analyzed cellular uptake of the complexes upon targeting to the tumor associated antigen c-erbB2 using the bi-functional protein sCAR-C6.5. In contrast with untargeted QDs (Figure 1A), targeted Ad-QD complexes were taken up by c-erbB2 expressing cells and clearly visible in intracellular compartments (Figure 1B). This indicates the potential of targeted Ad vectors to carry nanoparticles inside tumor cells, where they can function as imaging or therapeutic agents.

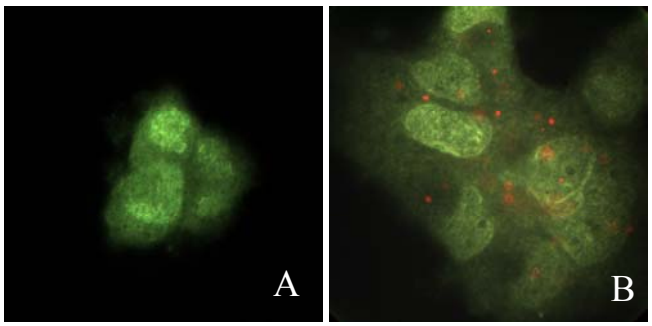

Figure 1: Dual Mode Fluorescence imaging of MDA-MB-361 cells, incubated with either (A) streptavidin-labeled QDs alone or (B) streptavidin-labeled QDs coupled to c-erbB2-targeted Ad vectors. Targeting the QDs to tumor cells utilizing the Ad platform resulted in a clear punctate pattern of red fluorescence, indicating their intracellular presence.

Next, we coupled Ni-NTA-labeled AuNPs to Ad vectors expressing a six-histidine tag in the hexon capsid protein. An increase of the density of Ad vectors in a CsCl gradient demonstrated the successful coupling of Ni-NTA-labeled AuNP to six-histidine labeled Ad vectors (Figure 2C, thick arrow), whereas the similar density of the control Ad (Figure 2A) and the Ad vector without a six-histidine tag but incubated with AuNP (Figure 2B), indicates that no unspecific interaction occurs between Ad and AuNP (thin arrow).

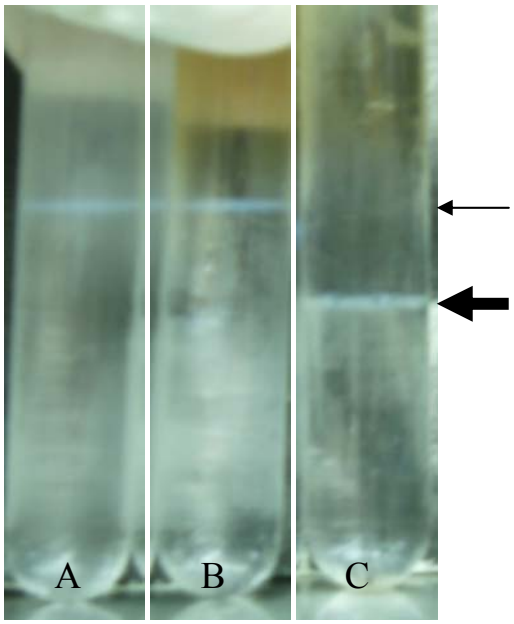

Figure 2: Photographs of CsCl density gradient centrifugation of (A) Ad vectors alone, (B), Ad vectors without a 6-His tag but with Ni-NTA AuNP and (C) Ad vectors labeled with 6-His in hexon, coupled to Ni-NTA-AuNP.

After successful coupling of AuNP to Ad vectors was demonstrated by the increase in density in a CsCl gradient, we analyzed the ability of the Ad vector to target the tumor-associated antigen carcinoembryonic antigen (CEA) and express the transgene it encodes.

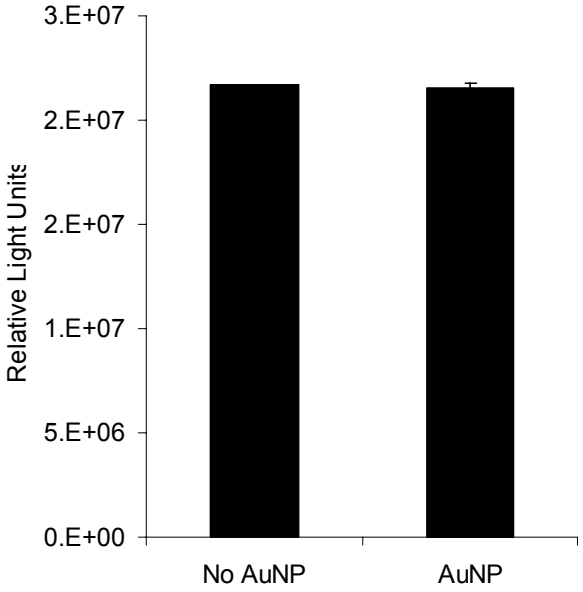

Figure 3: Luciferase expression in MC38-CEA-2 cells, 24 hours after infection by Ad vectors incorporating a 6-his tag in hexon, either without (left) or with (right) Ni-NTA AuNP coupled to their surface. Bars represent mean ± sd.

This is particularly important if gene therapy and nanotechnology will be used as synergistic therapeutic approaches within one multifunctional nanoscale system. Since the AuNPs were selectively coupled to the hexon capsid protein of the virus, which is not important for the viral retargeting and infection pathway, it was anticipated that transgene expression would not be reduced upon nanoparticle coupling. As expected, luciferase analysis indeed demonstrated that AuNP coupling to Ad did not negatively affect virus infectivity and retargeting ability to CEA-expressing MC38-CEA-2 cells (Figure 3). This is a significant improvement on coupling methods employed thus far, where AuNP were non-specifically coupled to lysine residues present in all capsid proteins, resulting in reduced infection and retargeting abilities of Ad at high ratios of AuNP:Ad [12].

4 CONCLUSION

The presented data demonstrates the feasibility of coupling metal nanoparticles to targeted Ad vectors. Importantly, Ad vector infectivity and retargeting ability were retained upon nanoparticle coupling. Therefore, Ad can provide a versatile platform for selective binding of nanoparticles, resulting in a multifunctional agent capable of simultaneous targeting and treatment of cancer by utilizing gene therapy and nanotechnology approaches. This will provide new opportunities for the diagnosis and treatment of tumors that are refractory to currently available classical therapeutic interventions.

5 ACKNOWLEDGEMENTS

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Adenoviral platform for selective assembly and targeted delivery of gold nanoparticles to tumor cells

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Nanotechnology holds great promise for the treatment of diseases like cancer. In this regard, gold nanoparticles (AuNPs) have biomedical applications such as drug delivery, imaging and hyperthermia induction. However, lack of AuNP targeting to tumor cells is a major impediment for realization of these therapeutic possibilities. Therefore, we propose to use targeted adenoviral (Ad) gene therapy vector as a platform for selective assembly and delivery of AuNPs to tumors. This would also allow a combination of gene therapy and nanotechnology for tumor treatment. We have previously demonstrated that AuNPs can be non-specifically coupled to Ad. We herein aim to further this paradigm by assembling AuNPs at specific Ad capsid locations and thus avoid detrimental effects on Ad infectivity and targeting that were observed with the non-specific approach. Towards this goal, 1.8 nm Ni-NTA-AuNPs were coupled to Ad vectors expressing a 6-His tag at different capsid locations, including fiber fibritin (FF, ~9 copies), pIX (240 copies) or hexon (720 copies). Upon coupling AuNPs to Ad, the molecular weight of the hexon virus increased in a CsCl density gradient indicating successful attachment of the AuNPs. However, no increase in the density of FF and pIX viruses was observed. Transmission electron microscopy confirmed the presence of gold in the hexon virus and its absence in FF and pIX viruses. This corroborates with the fact that that FF virus has few 6-His (~9) tags for AuNP binding. With respect to pIX, this protein is structurally located 65 Å below the main surface of the Ad capsid and might therefore be inaccessible to AuNPs. This indicates that hexon is the most optimal location for AuNP binding to the capsid. Importantly, no adverse effects on viral infectivity or tumor targeting ability were observed after coupling of AuNPs to the hexon virus. Therefore, Ad can provide a versatile platform for selective binding of AuNPs, resulting in a multifunctional agent capable of simultaneous targeting and treatment of cancer by utilizing gene therapy and nanotechnology approaches.

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Adenoviral Platform for Selective Assembly and Targeted Delivery of Gold Nanoparticles to Tumor Cells

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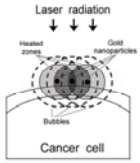
Abstract

Nanotechnology holds great promise for the treatment of diseases like cancer. In this regard, gold nanoparticles (AuNPs) have biomedical applications such as drug delivery, imaging and hyperthermia induction. However, lack of AuNP targeting to tumor cells is a major impediment for realization of these therapeutic possibilities. Therefore, we propose to use a targeted adenoviral (Ad) gene therapy vector as a platform for selective assembly and delivery of AuNPs to tumors. This would also allow a combination of gene therapy and nanotechnology for tumor treatment. We have previously demonstrated that AuNPs can be non-specifically coupled to Ad. We herein aim to further this paradigm by assembling AuNPs at specific Ad capsid locations and thus avoid detrimental effects on Ad infectivity and targeting that were observed with the non-specific approach. Towards this goal, 1.8 nm Ni-NTA-AuNPs were coupled to Ad vectors expressing a 6-His tag at different capsid locations, including fiber fibritin (FF; ~9 copies), pIX (240 copies) or hexon (720 copies). Upon coupling AuNPs to Ad, the density of the hexon virus increased in a CsCl density gradient indicating successful attachment of the AuNPs. However, no increase in the density of FF and pIX viruses was observed. Transmission electron microscopy confirmed the presence of gold in the hexon virus and its absence in FF and pIX viruses. This corroborates with the fact that that FF virus has few 6-His (~9) tags for AuNP binding. With respect to pIX, this protein is structurally located 65 Å below the main surface of the Ad capsid and might therefore be inaccessible to AuNPs. This indicates that hexon is the most optimal location for AuNP binding to the capsid. Importantly, no adverse effects on viral infectivity or tumor targeting ability were observed after coupling of AuNPs to the hexon virus. Therefore, Ad can provide a versatile platform for selective binding of AuNPs, resulting in a multifunctional agent capable of simultaneous targeting and treatment of cancer by utilizing gene therapy and nanotechnology approaches.

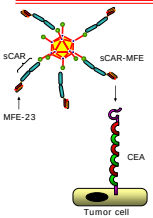
Gold Nanoparticles and Nanoclusters Can Kill Tumor Cells Upon Laser Irradiation



- Gold nanoparticles induce thermal bubble formation upon laser irradiation, resulting in hyperthermic cell killing
- Clustering of nanoparticles – nanoclusters – will result in overlapping bubbles, increasing therapeutic effects
- Targeting of nanoparticles or nanoclusters to tumor is necessary to achieve selective therapeutic effects

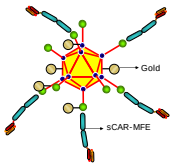
Zharov et al, Nanomed: Nanotech, Biol and Med, 2005, 1 (4): 326-345

Ad Vectors Can Be Targeted to Tumors



- Considerable progress has been made in targeting of adenoviral (Ad) vectors to tumors, using bi-functional adapter molecules
- sCAR-MFE is an example of an adapter molecule
  - sCAR binds to Ad knob
  - MFE-23 is a single chain antibody recognizing carcinoembryonic antigen (CEA)
- sCAR-MFE has shown *in vitro* and *in vivo* targeting abilities

Gold Nanoparticles Can Be Non-Specifically Coupled to Ad Vectors

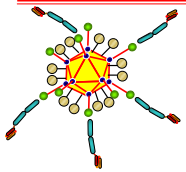


Tumor-Targeted Gold-Labeled Ad Vectors

- We have previously demonstrated that gold nanoparticles can be conjugated to Ad vectors using non-specific coupling strategies, forming nanoclusters
- Gold coupled to Ad vectors can be delivered to tumor cells
- However, non-specific gold coupling abrogated Ad infectivity and targeting at higher ratios of gold nanoparticles:Ad

Everts et al., Nano Letters, 2006, 6 (4): 587-591

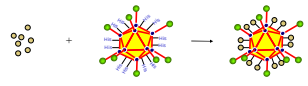
Hypothesis: Gold Nanoparticles Can be Selectively Coupled to Genetically Modified Ad Capsid Proteins



Tumor-Targeted Gold Nanoparticle-Labeled Ad Vector Using Selective Coupling

- Selective coupling of gold to the adenoviral capsid will be achieved using:
  - Genetically engineered Ad vectors expressing '6-His tags' at specific capsid locations
  - Gold nanoparticles with surface attached Ni-NTA groups
- Ni-NTA-Gold nanoparticles will selectively bind to Ad capsid proteins displaying 6-His
- Selective coupling will minimize the negative effects on Ad infectivity and targeting

Gold Nanoparticles Modified with Ni-NTA Can Be Specifically Coupled to 6-His Displaying Ad Vectors

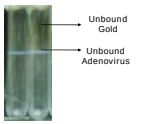


Ni-NTA-Gold nanoparticles

Adenovirus expressing 6-His tag in:
- fiber fibritin (FF, ~9 copies)
- pIX (240 copies)
- hexon (720 copies, pictured schematically above)

Gold nanoparticles coupled to adenovirus:
- FF
- pIX
- hexon (shown)

Gold Nanoparticles Do Not Bind Non-Specifically to Ad Vectors



Virus	Ad	Ad
Gold Nanoparticles	-	+

- As a negative control, Ni-NTA labeled AuNPs were incubated with Ad vectors that did not contain a 6-His tag and were loaded on a CsCl density gradient
- This virus band had the same density as the Ad vector that was not incubated with Ni-NTA-AuNPs
- This indicates that Ni-NTA-AuNPs, as expected, did not bind to Ad in the absence of 6-His sites

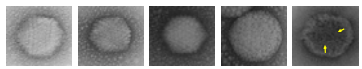
Gold Nanoparticle Binding to Ad Vectors is Dependent on Capsid Protein Locale

Virus	Ad	FF	pIX	hexon
Gold Nanoparticles	-	+	+	+

- Fiber fibritin (FF, ~9 available 6-His sites) has no significant change in density
  - Possible explanation is the limited number of 6-his sites
- pIX virus (240 available 6-His sites) may have a slight change in band density
  - Possible explanation is the limited surface accessibility of the pIX capsid protein
- Upon AuNP coupling, an increase in band density was observed for the hexon virus (720 available 6-His sites), as compared to the control Ad

Gold Nanoparticles Coupled to Ad Vectors Can Be Visualized by Transmission Electron Microscopy

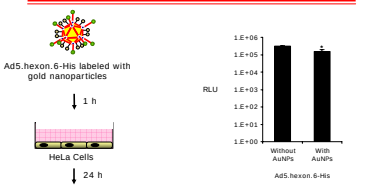
Magnification = 100,000 X



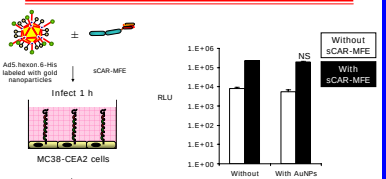
Virus	Ad5	Ad5	FF	pIX	hexon
6-His	-	-	+	+	+
Gold	-	+	+	+	+

- Absence of gold in Ad5 (control), FF, pIX
- Presence of gold in Ad with 6-His in hexon

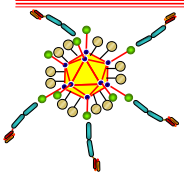
Adenoviruses with Selectively Coupled Gold Are Only Moderately Affected in Their Infectivity



Adenoviruses with Selectively Coupled Gold Can Be Targeted to CEA Expressing Cells



Targeted Adenoviruses with Selectively Coupled Gold Can Be Used For Tumor Treatment



Tumor-Targeted Gold Nanoparticle-Labeled Ad Vector Using Selective Coupling

- Gold nanoparticles (1.8 nm) can be selectively coupled to Ad vectors, forming nanoclusters
- Gold coupling is in accordance with the number and accessibility of binding sites on Ad capsid
- Gold coupling has only moderate effects on Ad infectivity
- Ad vectors can still be targeted to tumor cells after gold coupling
- Ad can serve as a platform for selective gold coupling and targeting to tumor cells resulting in novel multifunctional *viro-nano therapy agents* for tumor treatment

Saini et al., Nanomedicine: Nanotechnology, Biology, and Medicine, 2006, 200-206

Grant Acknowledgements: NIH P41RR04050 to Dr. Mark H. Ellisman, NIH R01CA111569, 5R01CA090547, DOD W81XWH-05-1-0035 to Dr. David T. Curiel, DOD W81XWH-06-1-0630 to Dr. Maaike Everts

Targeting Nanoparticles to Tumors using Adenoviral Vectors

V. Saini[1], M.R. Enervold[2], A. Perez[3], A. Koploy[3], G. Perkins[3], M.H. Ellisman[3], H.N. Green[4], S.B. Mirov[4], V.P. Zharov[5], M. Everts[6,7]

[1]Department of Physiology and Biophysics, [4]Department of Physics, [6]Division of Human Gene Therapy, Departments of Medicine, Surgery, Pathology and the Gene Therapy Center, [7]Division of Molecular and Cellular Pathology, Department of Pathology, University of Alabama at Birmingham; [2]CytoViva Inc., Auburn, Alabama; [3]National Center for Microscopy and Imaging Research, Center for Research in Biological Systems, School of Medicine, University of California, San Diego, [5]Philips Classic Laser Laboratories, University of Arkansas for Medical Sciences, Little Rock, Arkansas

Background/Objective: Despite advances in detection and treatment of cancer, development of novel therapies remains essential in the continuing battle against this disease; in this regard, nanotechnology holds great promise. For example, tumor imaging opportunities have expanded by the development of quantum dots (QDs) for fluorescence based detection, or magnetic nanoparticles for magnetic resonance imaging applications. Novel tumor treatment opportunities are exemplified by the use of gold nanoparticles, which upon absorption of laser energy radiate heat to kill neoplastic cells via hyperthermia. However, for all these applications of metal nanoparticles, selective tumor targeting is crucial for successful clinical application. Considering the great progress made in targeting adenoviral (Ad) gene therapy vectors to tumors, we therefore aim to couple metal nanoparticles with targeted Ad vectors in order to achieve specific, selective tumor accumulation. This combination of novel nanotechnology developments and gene therapy targeting strategies is expected to lead to the development of a unique methodology for cancer detection and treatment.

Hypothesis: Ad vectors can be conjugated with metal nanoparticles, without compromise of vector infectivity, targeting ability, or nanoparticle function.

Experimental Approach: For labeling Ad vectors with quantum dots, a chimeric virus expressing the biotin acceptor peptide in the hexon capsid protein was utilized.[1] This virus is metabolically biotinylated upon replication, facilitating interaction with streptavidin-labeled QDs (655 nm, Invitrogen). The Ad-QD complex was targeted to c-erbB2-expressing breast cancer cells (MDA-MB-361) using the previously described bi-functional adapter molecule sCAR-C6.5.[2] Cells were imaged utilizing the Dual Mode Fluorescence technique (CytoViva). For labeling Ad vectors with gold nanoparticles, an Ad containing a six-histidine motif in the hexon capsid protein[3] allowed for coupling of Ni-NTA-labeled gold nanoparticles (AuNP; Nanogold). AuNP-labeled Ad vectors were purified from remaining reagents using a CsCl density gradient. The purified Ad-AuNP complex was targeted to CEA-expressing colon cancer cells (MC38-CEA-2) using the previously described bi-functional adapter molecule sCAR-MFE.[4] Virus infectivity and targeting ability were determined using luciferase transgene expression analysis of the infected cells.

Results: Targeted Ad-QD were taken up by c-erbB2 expressing cells and clearly visible as multiple fluorescent spots in intracellular compartments (Fig. 1). An increase in the density of Ad vectors in a CsCl gradient demonstrated a successful coupling reaction of Ni-NTA-labeled AuNP to six-histidine labeled Ad vectors (Fig. 2). Luciferase analysis demonstrated that AuNP coupling to Ad did not negatively affect virus infectivity and retargeting ability to CEA expressing cells (Fig. 3).

Discussion/Impact/Significance: The presented data demonstrates the feasibility of coupling metal nanoparticles to targeted Ad vectors. Importantly, Ad vector infectivity and retargeting ability in addition to nanoparticles utility remained unaffected. Therefore, Ad can provide a versatile platform for selective binding of nanoparticles, resulting in a multifunctional agent capable of simultaneous targeting and treatment of cancer through utilization of gene therapy and nanotechnology approaches. This will provide new opportunities for advanced diagnosis and treatment of tumors refractory to the currently available classical therapeutic interventions.

Topic area: CancerNano 2007: Drug Delivery

References

1 Campos SK, Barry MA. Comparison of adenovirus fiber, protein IX, and hexon capsomeres as scaffolds for vector purification and cell targeting. *Virology* 2006; **349:** 453-462.

2 Kashentseva EA, Seki T, Curiel DT, Dmitriev IP. Adenovirus targeting to c-erbB-2 oncoprotein by single-chain antibody fused to trimeric form of adenovirus receptor ectodomain. *Cancer Res* 2002; **62:** 609-616.

3 Wu H *et al.* Identification of sites in adenovirus hexon for foreign peptide incorporation. *J Virol* 2005; **79:** 3382-3390.

4 Everts M *et al.* Selective induction of tumor-associated antigens in murine pulmonary vasculature using double-targeted adenoviral vectors. *Gene Ther* 2005; **12:** 1042-1048.

Figures

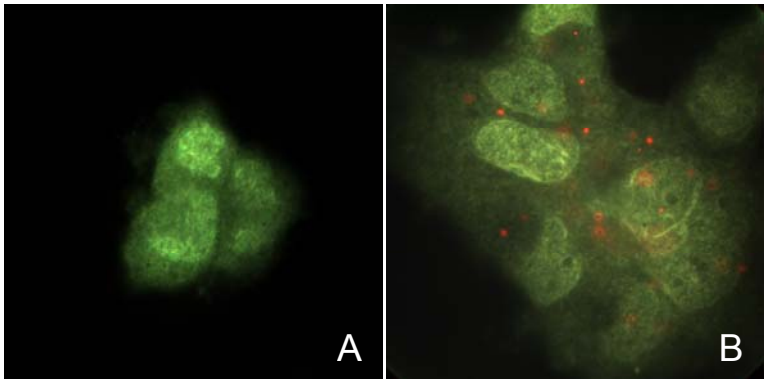


Fig. 1 Dual-mode fluorescence imaging of MDA-MB-361 cells, incubated for 30 min at 4 °C with either (A) streptavidin-labeled QDs alone or (B) streptavidin-labeled QDs conjugated to c-erbB2-targeted Ad vectors. After the initial incubation step, cells were washed and incubated in cell culture medium for an additional 30 min at 37 °C, to allow Ad binding and internalization. Targeting the QDs to tumor cells utilizing the Ad platform resulted in a clear punctate pattern of red fluorescence, indicating their intracellular presence.

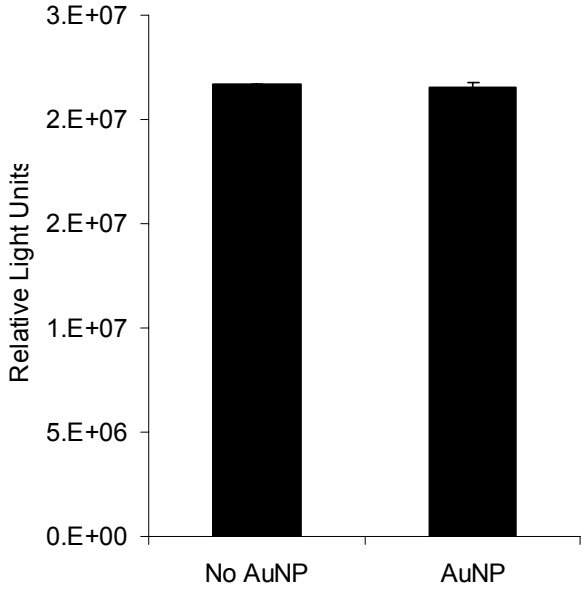


Fig. 3 Luciferase expression in MC38-CEA-2 cells, 24 hours after infection by Ad vectors incorporating a 6-his tag in hexon, either without (left) or with (right) Ni-NTA AuNP coupled to their surface. Bars represent mean ± sd. Transgene expression is not affected by AuNP coupled to the surface of the vectors, feasibilizing delivery of AuNP and gene therapy as a combinatorial therapeutic approach.

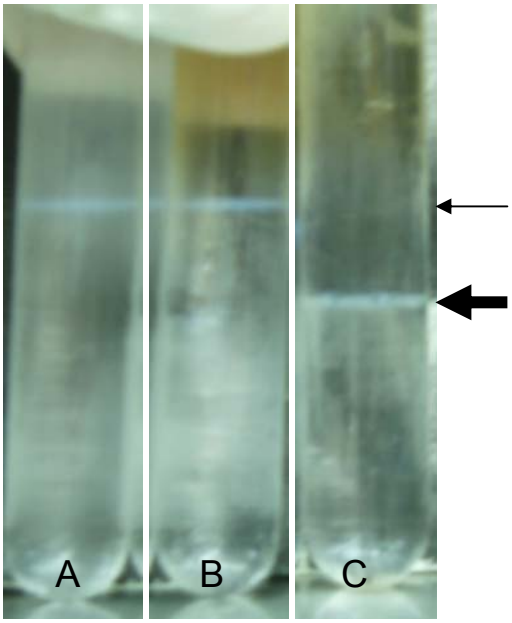


Fig. 2 Photographs of CsCl density gradient centrifugation of (A) Ad vectors alone, (B), Ad vectors without a 6-His tag but with Ni-NTA AuNP and (C) Ad vectors labeled with 6-His in hexon, coupled to Ni-NTA AuNP. Note the similar density of Ad bands in (A) and (B), indicating that no unspecific interaction occurs between Ad and AuNP (thin arrow). In contrast, Ad vectors labeled with 6-his in hexon clearly increased in density (C), indicating successful coupling of AuNP.

Targeting Nanoparticles to Tumors using Adenoviral Vectors

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[1]Department of Physiology and Biophysics, [4]Department of Physics, [6]Division of Human Gene Therapy, Departments of Medicine, Surgery, Pathology and the Gene Therapy Center, [7]Division of Molecular and Cellular Pathology, Department of Pathology, University of Alabama at Birmingham; [2]CytoViva Inc., Auburn, Alabama; [3]National Center for Microscopy and Imaging Research, Center for Research in Biological Systems, School of Medicine, University of California, San Diego; [5]Philips Classic Laser Laboratories, University of Arkansas for Medical Sciences, Little Rock, Arkansas

Summary

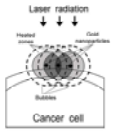
Despite advances in detection and treatment of cancer, development of novel therapies remains essential; in this regard, nanotechnology holds great promise. For example, tumor imaging opportunities have expanded by the development of quantum dots (QDs) for fluorescence based detection, and novel tumor treatment opportunities are exemplified by the use of gold nanoparticles (AuNPs). AuNP will, upon absorption of laser energy, radiate heat to kill neoplastic cells via hyperthermia. However, for all these applications of metal nanoparticles, selective tumor targeting is crucial for successful clinical application. Considering the great progress made in targeting adenoviral (Ad) gene therapy vectors to tumors, we therefore aim to couple metal nanoparticles with targeted Ad vectors in order to achieve specific, selective tumor accumulation. We herein demonstrate that metal nanoparticles such as QDs and AuNPs can indeed be coupled to adenoviral vectors, without compromising viral infectivity, retargeting ability or function of the nanoparticles. This combination of novel nanotechnology developments and gene therapy targeting strategies is expected to lead to the development of a unique methodology for cancer detection and treatment.

1

Nanoparticles Can Be Used for Imaging and Treatment of Cancer

Example: magnetic nanoparticles (FePt) suitable for MRI

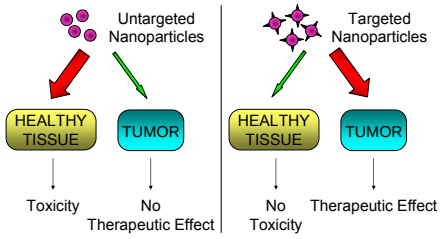
Example: AuNP for hyperthermia induction upon laser irradiation



- Nanotechnology has promise for imaging and therapy of tumors
- Examples include:
  - Quantum Dots
    - Imaging - Fluorescence
  - Magnetic Nanoparticles
    - Imaging – MRI
    - Therapy – Hyperthermia induction upon exposure to magnetic fields
  - Gold Nanoparticles (AuNP)
    - Therapy – Hyperthermia induction upon laser irradiation

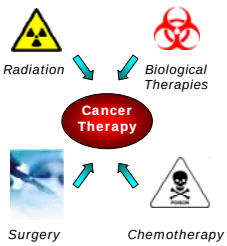
2

Targeting of Nanoparticles is Needed to Achieve Necessary Selectivity



3

Combination of Multiple Therapies Often Most Effective for Tumor Treatment

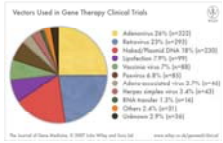


- In clinical practice, multiple treatment options are often used simultaneously
  - Radiation therapy
  - Biological therapies such as gene therapy
  - Surgery
  - Chemotherapy
- Therapies are able to work synergistically, thereby improving tumor treatment

4

Adenovirus Is a Suitable Vector for Cancer Gene Therapy

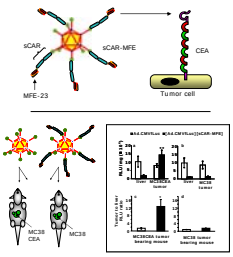
Adenovirus (Ad)



- Gene therapy is the use of genetic material to modify a patient's cells for the treatment of an inherited or acquired disease
- Non-viral and viral vectors are used to deliver the genetic material inside target cells
- Adenovirus is an example of a viral vector and has several advantages
  - Ability to infect a wide range of cell types
  - High levels of transgene expression
  - Efficient methods to generate recombinant viruses

5

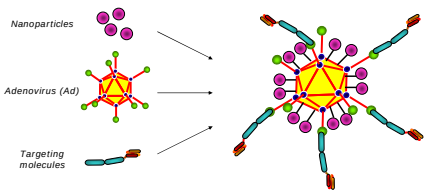
Ad Can Be Targeted to Tumors Using Adapter Molecules



- Adapter molecules are bifunctional proteins
  - A domain recognizing the adenoviral vector
  - A domain recognizing the target receptor
- sCAR-MFE is an example of an adapter molecule
  - sCAR binds to Ad knob
  - MFE-23 is a single chain antibody recognizing carcinoembryonic antigen (CEA)
- sCAR-MFE is able to target Ad to hepatic tumors after systemic administration (*Li et al, Cancer Res., in press*)

6

Hypothesis



Tumor-targeted Ad vectors can serve as a delivery platform for metal nanoparticles and thus provide a novel method for cancer imaging and combination therapy.

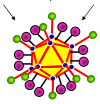
7

Strategies for Coupling Nanoparticles to Ad Vectors

Genetically engineered Ad with binding tag in the capsid protein hexon

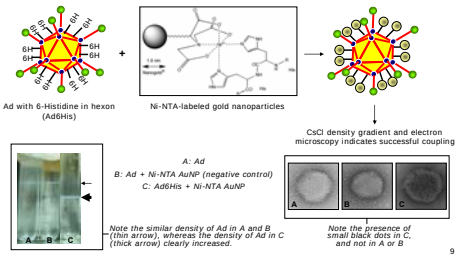
Nanoparticles with reactive group on their surface

Nanoparticles coupled to Ad vectors



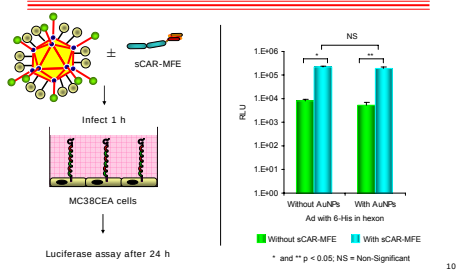
8

Gold Nanoparticles Can Be Coupled to Ad Vectors



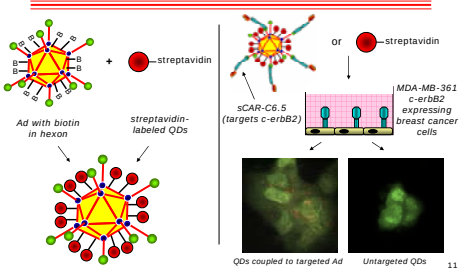
9

Targeting of Ad Vectors Is Retained Upon Coupling of Gold Nanoparticles



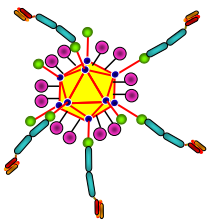
10

Quantum Dots Can Be Coupled to Ad Vectors and Targeted to Tumor Cells



11

Targeted Ad as a Multifunctional Platform for Nanoparticle Delivery to Tumors



- Metal nanoparticles such as gold nanoparticles or quantum dots can be coupled to targeted Ad vectors
- Retargeting ability of Ad using bifunctional adapter molecules is not affected upon coupling of nanoparticles
- This feasibilizes a combination approach of nanotechnology and gene therapy for targeting, imaging and therapy of cancer

12

Grant Acknowledgements: NIH P41RR04050 to Dr. Mark H. Ellisman and DOD W81XWH-06-1-0630 to Dr. Maaike Everts

Determining Parameters for Using Gold Nanoparticles for Hyperthermia Treatment in Tumor Cells

Victoria D. Towner[5], Vaibhav Saini[1,2], Dmitri V. Martyshkin[3], Sergei B. Mirov[3], Maaike Everts[1,4]

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Nanotechnology offers novel treatment opportunities for diseases like cancer. In this regard, gold nanoparticles (AuNPs) can be used for hyperthermic tumor cell killing. However, targeted delivery of AuNPs to tumors limits the utilization of this treatment option. In this regard, we have previously demonstrated successful coupling of 1.8 nm diameter AuNPs to tumor-targeted gene therapeutic adenoviral (Ad) vectors. The AuNP-labeled Ad vector represents a multifunctional nanoscale system that can be utilized for cancer treatment through nanotechnology and gene therapy approaches. In this study, we aim to further delineate the parameters required for laser-induced AuNP-mediated hyperthermic tumor cell ablation, which would pave the way for combining hyperthermia with gene therapy. Towards this goal, we laser irradiated (5.2 W, 532 nm, 600 pulses) HeLa cells incubated with AuNPs (1.8 nm diameter), stained the cells with propidium iodide, and subjected them to flow cytometry viability analysis. However, the results of the analysis did not show any difference in cell viability in either absence or presence of AuNPs. The lack of hyperthermic induction might be due to weak absorption in the 532 nm range by the 1.8 nm diameter AuNPs. Thus, we are currently testing a range of AuNPs (5 nm, 20 nm, and 40 nm diameter) to determine the optimum size for inducing hyperthermia. Upon optimization of the critical parameters for hyperthermic induction using AuNPs, it would be possible to combine nanotechnology with gene therapy for tumor treatment.

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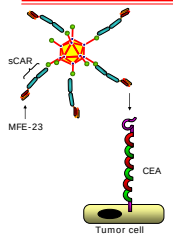
1

Abstract

Nanotechnology offers novel treatment opportunities for diseases like cancer. In this regard, gold nanoparticles (AuNPs) can be used for hyperthermic tumor cell killing. However, the lack of targeted delivery of AuNPs to tumors limits the utilization of this treatment option. Towards this end, we have previously demonstrated successful coupling of 1.8 nm diameter AuNPs to tumor-targeted gene therapeutic adenoviral (Ad) vectors. The AuNP-labeled Ad vector represents a multifunctional nanoscale system that can be utilized for cancer treatment through nanotechnology and gene therapy approaches. In this study, we aim to further delineate the parameters required for laser-induced AuNP-mediated hyperthermic tumor cell ablation, which would pave the way for combining hyperthermia with gene therapy. Towards this goal, we laser irradiated (5.2 W, 532 nm, 600 pulses) HeLa cells incubated with AuNPs (1.8 nm diameter), stained the cells with propidium iodide, and subjected them to flow cytometry viability analysis. However, the results of the analysis did not show any difference in cell viability in either absence or presence of AuNPs. The lack of hyperthermic induction might be due to weak absorption in the 532 nm range by the 1.8 nm diameter AuNPs. Thus, we are currently testing a range of AuNPs (5 nm, 20 nm, and 40 nm diameter) to determine the optimum size for inducing hyperthermia. Upon optimization of the critical parameters for hyperthermic induction using AuNPs, it would be possible to combine nanotechnology with gene therapy for tumor treatment.

1

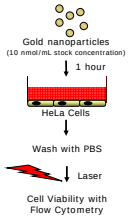
Ad Gene Therapy Vector for Targeting Gold Nanoparticles to Tumors



- Adenoviral (Ad) vectors can be targeted to tumors, using bi-functional adapter molecules
- sCAR-MFE is an example of an adapter molecule
  - sCAR binds to Ad knob
  - MFE-23 is a single chain antibody recognizing carcinoembryonic antigen (CEA)
- sCAR-MFE has shown *in vitro* and *in vivo* targeting abilities
- This opens the possibility of Ad vectors being used to target gold nanoparticles to tumor cells

4

Experimental Design



- 1.8 nm diameter nanoparticles were incubated with HeLa cells for 1 hour, then washed with PBS
- The cells were laser irradiated (5.2 W, 532 nm, 600pulses)
- The cells were stained with propidium iodide and subjected to flow cytometry viability analysis

7

Conclusion and Future Plans

- 1.8 nm gold nanoparticles do not absorb well at 532 nm, making it unsuitable for hyperthermic induction
- Future focus will be on 5 nm, 20 nm, and 40 nm gold nanoparticles

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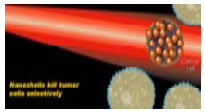
Nanotechnology Offers Novel Treatment Opportunities

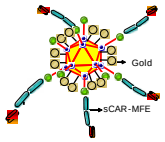


Source: http://nano.cancer.gov/resource_center/nanotech_nanoshells.asp

- Nanoparticles have novel properties which can be harnessed for unique biomedical applications
- In this regard, nanoparticles can be used for:
  - Imaging
  - Hyperthermia

2

Gold Nanoparticles Can Be Coupled to Ad Vectors



Tumor-Targeted
Gold Nanoparticle-Labeled Ad Vector

- We have previously demonstrated that 1.8 nm gold nanoparticles can be conjugated to Ad vectors
- Gold coupled to Ad vectors can be delivered to tumor cells
- The next step is to induce hyperthermic tumor cell ablation using tumor-targeted gold nanoparticle labeled Ad vectors

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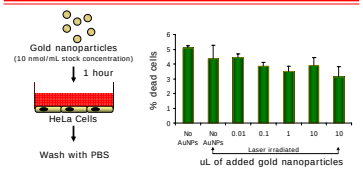
Using 1.8nm AuNPs in Laser Irradiation Does Not Significantly Change Cell Viability



- No difference in cell viability after hyperthermia in the different treatment groups

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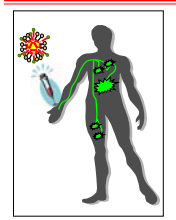
Determining Parameters for Hyperthermic Induction Can Offer New Cancer Treatments



- Hyperthermia induction can be the bridge to link nanotechnology with gene therapy for cancer treatment

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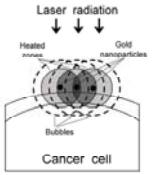
Gold Nanoparticles and Nanoclusters Can Kill Tumor Cells Upon Laser Irradiation

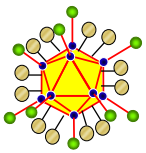


Zharov et al,
Nanomed: Nanotech, Biol and Med,
2005, 1 (4); 326-345

- Gold nanoparticles induce thermal bubble formation upon laser irradiation, resulting in hyperthermic cell killing
- Clustering of nanoparticles – nanoclusters – will result in overlapping bubbles, increasing therapeutic effects
- Impediments of realization of this tumor treatment:
  - Delivery to tumor cells
  - Delineating parameters for hyperthermia induction

3

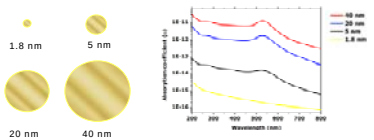
Delineating Parameters for Use of Gold Nanoparticles for Hyperthermic Induction



- Our initial experiments used 1.8 nm gold nanoparticles for coupling to Ad vectors
- We therefore used this size nanoparticle to delineate parameters for tumor-targeted gold nanoparticle-labeled Ad vectors

6

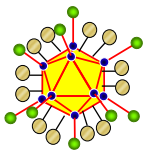
Various Sizes of Gold Nanoparticles Absorb Differently at the 532 nm Wavelength



- The 1.8 nm nanoparticle does not absorb well at 532 nm, which explains the lack of hyperthermic induction

9

Realization of Nanoparticle Parameters



- Determining the optimum-sized nanoparticle to use with an Ad vector is one more step towards combining gene therapy and nanotechnology

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Grant Acknowledgements: This study was supported by the following to Dr. M. Everts: DOD W81XWH-06-1-0630, The National Cancer Institute CA13148-35 UAB Comprehensive Cancer Center Junior Faculty Development Grant Program, and the UAB Center for Women's Reproductive Health Pilot and Feasibility Program

Targeting Nanoparticles to Tumors using Adenoviral Vectors

J.M. Warren[1], R.G. Beam[1], V. Saini[2,3], M.R. Enervold[4], A. Perez[5], G. Perkins[5], M.H. Ellisman[5], M. Everts[3,6]

[1]Department of Chemistry, [2]Department of Physiology and Biophysics, [3]Division of Human Gene Therapy, Departments of Medicine, Surgery, Pathology and the Gene Therapy Center, [6]Division of Molecular and Cellular Pathology, Department of Pathology, University of Alabama at Birmingham; [4]CytoViva Inc., Auburn, Alabama; [5]National Center for Microscopy and Imaging Research, Center for Research in Biological Systems, School of Medicine, University of California, San Diego

Development of novel therapies remains essential for treatment of cancer; in this regard, nanotechnology holds great promise. For example, tumor imaging opportunities have expanded by the development of quantum dots (QDs), and novel tumor treatment opportunities are exemplified by the use of gold nanoparticles (AuNPs). However, for all these applications of metal nanoparticles, selective tumor targeting is crucial for successful clinical application. Considering the progress made in targeting adenoviral (Ad) gene therapy vectors to tumors, we aim to couple metal nanoparticles to targeted Ad vectors to achieve selective tumor accumulation. We have demonstrated that metal nanoparticles such as QDs and AuNPs can indeed be coupled to Ad vectors, without compromising viral infectivity, retargeting ability or function of the nanoparticles. This innovative combination strategy is therefore expected to lead to the development of a unique methodology for cancer detection and treatment.

Targeting Nanoparticles to Tumors using Adenoviral Vectors

V. Saini[1], M.R. Enervold[2], A. Perez[3], A. Koploy[3], G. Perkins[3], M.H. Ellisman[3], H.N. Green[4], S.B. Mirov[4], V.P. Zharov[5], M. Everts[6,7]

[1]Department of Physiology and Biophysics, [4]Department of Physics, [6]Division of Human Gene Therapy, Departments of Medicine, Surgery, Pathology and the Gene Therapy Center, [7]Division of Molecular and Cellular Pathology, Department of Pathology, University of Alabama at Birmingham; [2]CytoViva Inc., Auburn, Alabama; [3]National Center for Microscopy and Imaging Research, Center for Research in Biological Systems, School of Medicine, University of California, San Diego; [5]Philips Classic Laser Laboratories, University of Arkansas for Medical Sciences, Little Rock, Arkansas

Summary

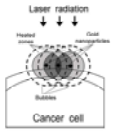
Despite advances in detection and treatment of cancer, development of novel therapies remains essential; in this regard, nanotechnology holds great promise. For example, tumor imaging opportunities have expanded by the development of quantum dots (QDs) for fluorescence based detection, and novel tumor treatment opportunities are exemplified by the use of gold nanoparticles (AuNPs). AuNP will, upon absorption of laser energy, radiate heat to kill neoplastic cells via hyperthermia. However, for all these applications of metal nanoparticles, selective tumor targeting is crucial for successful clinical application. Considering the great progress made in targeting adenoviral (Ad) gene therapy vectors to tumors, we therefore aim to couple metal nanoparticles with targeted Ad vectors in order to achieve specific, selective tumor accumulation. We herein demonstrate that metal nanoparticles such as QDs and AuNPs can indeed be coupled to adenoviral vectors, without compromising viral infectivity, retargeting ability or function of the nanoparticles. This combination of novel nanotechnology developments and gene therapy targeting strategies is expected to lead to the development of a unique methodology for cancer detection and treatment.

1

Nanoparticles Can Be Used for Imaging and Treatment of Cancer

Example: magnetic nanoparticles (FePt) suitable for MRI

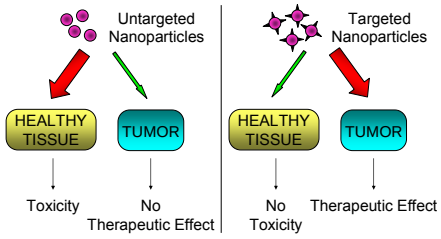
Example: AuNP for hyperthermia induction upon laser irradiation



- Nanotechnology has promise for imaging and therapy of tumors
- Examples include:
  - Quantum Dots
    - Imaging - Fluorescence
  - Magnetic Nanoparticles
    - Imaging – MRI
    - Therapy – Hyperthermia induction upon exposure to magnetic fields
  - Gold Nanoparticles (AuNP)
    - Therapy – Hyperthermia induction upon laser irradiation

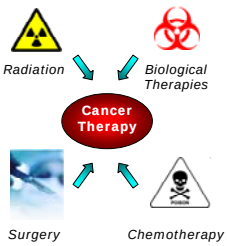
2

Targeting of Nanoparticles is Needed to Achieve Necessary Selectivity



3

Combination of Multiple Therapies Often Most Effective for Tumor Treatment

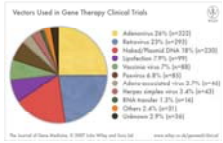


- In clinical practice, multiple treatment options are often used simultaneously
  - Radiation therapy
  - Biological therapies such as gene therapy
  - Surgery
  - Chemotherapy
- Therapies are able to work synergistically, thereby improving tumor treatment

4

Adenovirus Is a Suitable Vector for Cancer Gene Therapy

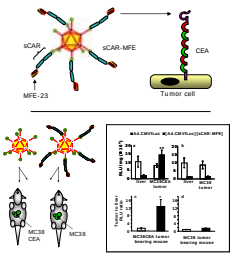
Adenovirus (Ad)



- Gene therapy is the use of genetic material to modify a patient's cells for the treatment of an inherited or acquired disease
- Non-viral and viral vectors are used to deliver the genetic material inside target cells
- Adenovirus is an example of a viral vector and has several advantages
  - Ability to infect a wide range of cell types
  - High levels of transgene expression
  - Efficient methods to generate recombinant viruses

5

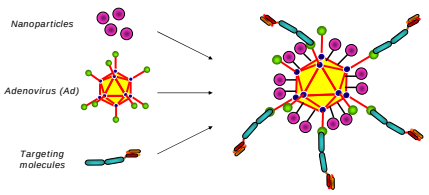
Ad Can Be Targeted to Tumors Using Adapter Molecules



- Adapter molecules are bifunctional proteins
  - A domain recognizing the adenoviral vector
  - A domain recognizing the target receptor
- sCAR-MFE is an example of an adapter molecule
  - sCAR binds to Ad knob
  - MFE-23 is a single chain antibody recognizing carcinoembryonic antigen (CEA)
- sCAR-MFE is able to target Ad to hepatic tumors after systemic administration (*Li et al, Cancer Res., in press*)

6

Hypothesis



Tumor-targeted Ad vectors can serve as a delivery platform for metal nanoparticles and thus provide a novel method for cancer imaging and combination therapy.

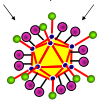
7

Strategies for Coupling Nanoparticles to Ad Vectors

Genetically engineered Ad with binding tag in the capsid protein hexon

Nanoparticles with reactive group on their surface

Nanoparticles coupled to Ad vectors



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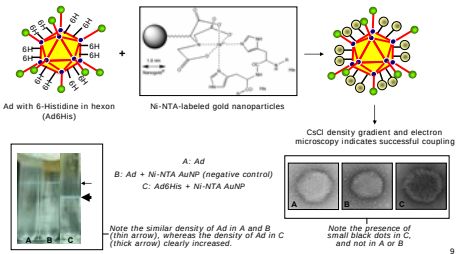
Gold Nanoparticles Can Be Coupled to Ad Vectors



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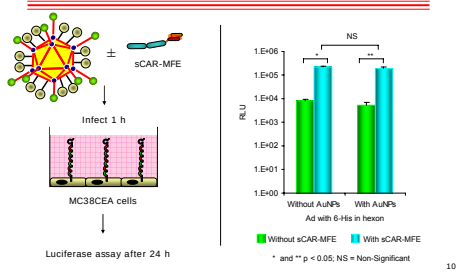
Targeting of Ad Vectors Is Retained Upon Coupling of Gold Nanoparticles



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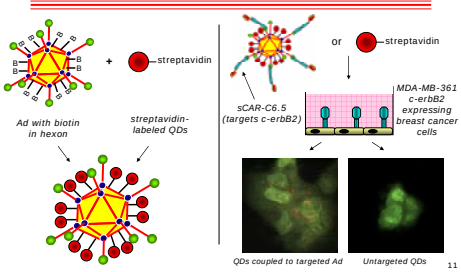
Quantum Dots Can Be Coupled to Ad Vectors and Targeted to Tumor Cells



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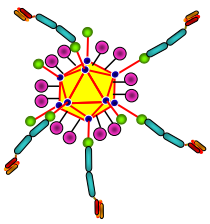
Targeted Ad as a Multifunctional Platform for Nanoparticle Delivery to Tumors



- Metal nanoparticles such as gold nanoparticles or quantum dots can be coupled to targeted Ad vectors
- Retargeting ability of Ad using bifunctional adapter molecules is not affected upon coupling of nanoparticles
- This feasibilizes a combination approach of nanotechnology and gene therapy for targeting, imaging and therapy of cancer

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Grant Acknowledgements: NIH P41RR04050 to Dr. Mark H. Ellisman and DOD W81XWH-06-1-0630 to Dr. Maaike Everts

Adenovirus as a platform for selective assembly and targeted delivery of gold nanoparticles to tumor cells

V. Saini[1], D. V. Martyshkin[1], S. B. Mirov[1], V. D. Towner[1], H. Wu[1], L. Pereboeva[1], A. Borovjagin[1], A. Perez[2], A. Koploy[2], G. Perkins[2], M.H. Ellisman[2], D.T. Curiel[1], M. Everts[1]

[1]University of Alabama at Birmingham (UAB), Birmingham, [2]Center for Research in Biological Systems, School of Medicine, University of California, San Diego

Nanotechnology holds great promise for the treatment of diseases like cancer. In this regard, gold nanoparticles (AuNPs) have biomedical applications such as drug delivery, imaging, and hyperthermic tumor cell ablation. However, lack of AuNP targeting to tumor cells is a major impediment for realization of these therapeutic possibilities. Therefore, we propose to use targeted adenoviral (Ad) gene therapy vector as a platform for selective assembly and specific delivery of AuNPs to tumors. This would also allow a combination of gene therapy and nanotechnology for the treatment of cancer. We have previously demonstrated that AuNPs can be non-specifically coupled to Ad vectors. We herein aim to further this paradigm by coupling AuNPs to specific Ad capsid locations and thus avoiding the detrimental effects on Ad infectivity and targeting that were observed with the non-specific approach. Towards this goal, 1.8 nm Ni-NTA-AuNPs were coupled to Ad vectors expressing a 6-His tag at different capsid locations, including fiber fibritin (FF, ~9 copies), pIX (240 copies) or hexon (720 copies). Upon coupling AuNPs to Ad, the molecular weight of the hexon virus increased in a CsCl density gradient indicating successful attachment of the AuNPs. However, no increase in the density of FF and pIX viruses was observed. Transmission electron microscopy confirmed the presence of AuNPs in the hexon virus and its absence in the FF and the pIX viruses. This corroborates with the fact that that FF virus has few 6-His (~9) tags for AuNP binding. With respect to pIX, this protein is structurally located 65 Å below the main surface of the Ad capsid and might therefore be inaccessible to AuNPs. Atomic absorption spectroscopy was utilized to determine that 56±4 AuNPs were bound per hexon virus. This indicates that hexon is the most optimal location for AuNP binding to the capsid. Also, specifically AuNP-labeled Ad vector infectivity for native Ad vector receptor expressing HeLa cells was 2-fold higher as compared to the non-specifically labeled vectors. Importantly, unlike the non-specifically AuNP-labeled Ad vectors, no adverse effects on Ad vector tumor targeting ability were observed after specific coupling of AuNPs to the hexon virus. Therefore, Ad can provide a versatile platform for selective assembly of AuNPs, resulting in a multifunctional agent capable of simultaneous targeting and treatment of cancer by utilizing gene therapy and nanotechnology approaches.

Targeting Quantum Dots to Tumors using Adenoviral Vectors

J.M. Warren[1], R.G. Beam[1], V. Saini[2,3], M. Everts[3,4]

[1]Department of Chemistry, [2]Department of Physiology and Biophysics, [3]Division of Human Gene Therapy, Departments of Medicine, Surgery, Pathology and the Gene Therapy Center, [4]Division of Molecular and Cellular Pathology, Department of Pathology, University of Alabama at Birmingham

Background/Objective: Despite advances in detection and treatment of cancer, development of novel therapies remains essential in the continuing battle against this disease; in this regard, nanotechnology holds great promise. For example, tumor imaging opportunities have expanded by the development of quantum dots (QDs) for fluorescence based detection, or magnetic nanoparticles for magnetic resonance imaging applications. However, for all these applications of metal nanoparticles, selective tumor targeting is crucial for successful clinical application. Considering the great progress made in targeting adenoviral (Ad) gene therapy vectors to tumors, we therefore aim to couple QDs with targeted Ad vectors in order to achieve specific, selective tumor accumulation. This combination of novel nanotechnology developments and gene therapy targeting strategies is expected to lead to the development of a unique methodology for cancer detection and treatment.

Hypothesis: Ad vectors can be conjugated with QDs, without compromise of vector infectivity, targeting ability, or nanoparticle function.

Experimental Approach: For labeling Ad vectors with quantum dots, a chimeric virus expressing the biotin acceptor peptide in the hexon capsid protein was utilized.[1] This virus is metabolically biotinylated upon replication, facilitating interaction with streptavidin-labeled QDs (655 nm, Invitrogen). The Ad-QD complex was targeted to c-erbB2-expressing breast cancer cells (AU-565) using the previously described bi-functional adapter molecule sCAR-C6.5.[2] Cells were imaged utilizing Confocal Laser Scanning Microscopy.

Results: Targeted Ad-QDs were taken up by c-erbB2 expressing cells and clearly visible as multiple fluorescent spots in intracellular compartments. In contrast, QDs were not taken up by themselves, nor when coupled to non-targeted Ad vectors.

Discussion/Conclusion: The presented data demonstrates the feasibility of coupling metal nanoparticles such as QDs to targeted Ad vectors. Importantly, Ad retargeting ability upon addition of the nanoparticles remained unaffected. Therefore, Ad can provide a versatile platform for selective binding of nanoparticles, resulting in a multifunctional agent capable of simultaneous targeting and treatment of cancer through utilization of gene therapy and nanotechnology approaches. This will provide new opportunities for advanced diagnosis and treatment of tumors refractory to the currently available classical therapeutic interventions.

1 Campos SK, Barry MA. Comparison of adenovirus fiber, protein IX, and hexon capsomeres as scaffolds for vector purification and cell targeting. *Virology* 2006; **349:** 453-462.

2 Kashentseva EA, Seki T, Curiel DT, Dmitriev IP. Adenovirus targeting to c-erbB-2 oncoprotein by single-chain antibody fused to trimeric form of adenovirus receptor ectodomain. *Cancer Res* 2002; **62:** 609-616.